Bioprocess Engineering for the Application of P450s

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Publication date:
2015

Document Version
Publisher's PDF, also known as Version of record

DTU

2015

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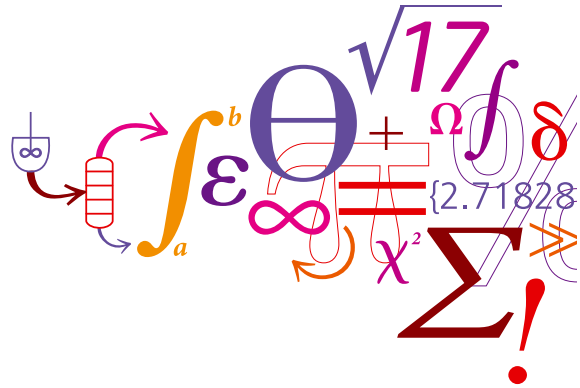

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Ph.D. Thesis
Jan 2015

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ISBN : 978-87-93054-76-9

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Print: J&R Frydenberg A/S
København
August 2015
ISBN: 978-87-93054-76-9

Abstract

Biocatalytic processes are advancing because of their high selectivity and mild operating conditions, in contrast to many chemical catalyzed processes. This is a clear advantage and frequently results in improved environmental performance. Biocatalytic processes have been implemented replacing traditional chemical catalysts as well as enabling new synthesis. Regardless of the process routes, the economic feasibility is crucial for successful industrial implementation. This has also been demonstrated by implemented biocatalytic processes, showing a clear cost advantage compared to the chemical alternative.

One family of enzymes described to have a lot of potential for industrial biocatalysis is cytochrome P450 monooxygenases. The main motivation for this statement is their ability to hydroxylate non-activated hydrocarbons in a specific manner, using molecular oxygen as oxidant. Containing more than 26 000 enzymes, this family includes diverse enzymes from all kingdoms of life. However, their dependence on cofactor, redox partners and relatively low activity and stability hinders the development of efficient processes. In this thesis, a novel systematic approach has been applied to identify bottlenecks for economically feasible whole cell P450 catalyzed processes to direct research and enable faster implementation. A methodological approach was introduced by reviewing literature based on guidance by economic metrics, followed by cases studies to confirm the initial analysis. The last part of the thesis consists of an economic assessment based on a process model using experimentally gained knowledge, including a sensitivity analysis of the biological parameters protein expression and enzyme total turnover.

Case studies of various complexities have been chosen throughout the thesis. The first case study was performed using a P450 fusion construct expressed in the well explored host *Escherichia coli* performing ω-hydroxylation of dodecanoic acid. This system represents an artificial fusion construct in a non-natural P450 expressing host. The main limitations in this case were identified to be the

stability and activity of the P450, cofactor regeneration by the host cell and substrate inhibition. The latter was partially circumvented by the introduction of substrate in solid form. The second case study utilized a naturally expressing P450 host, *Bacillus megaterium*, expressing the steroid hydroxylase CYP106A2 for 15β-hydroxylation of cyproterone acetate. The catalytic activity of the overexpressed CYP106A2 was dependent on the natural redox partners in the host cell. The stability of the P450 was also here identified as one of the limitations as well as product inhibition. Product inhibition was in this case addressed by introducing a modified β-cyclodextrin, yielding 98 % conversion in the gram scale.

P450 catalyzed whole cell processes have been identified suitable for production of high value molecules. The main limitations have been shown to be P450 stability and activity, substrate and product inhibition and cofactor regeneration of heterologous expression host. Furthermore, growing cells, where fermentation and biocatalysis is performed in one step is shown to be the most economically feasible option.

Dansk resumé

Betydningen af biokatalytiske processers forøges på grund af deres høje selektivitet og milde reaktionsbetingelser sammenlignet med mange kemisk katalyserede processer. Dette er en åbenlys fordel, hvilket ofte også resulterer i en reducering af miljøpåvirkningen. Biokatalytiske processer bliver implementeret for at erstatte traditionelle kemiske katalysatorer eller for at åbne op for nye synteseveje. Den økonomiske rentabilitet er, uanset processen, afgørende for succesfuld implementering på industrielskala. Dette er også demonstreret i allerede implementerede biokatalytiske processer, der viser en klar økonomisk fordel sammenlignet med de kemiske alternativer.

Cytochrome P450 monooxygenases er en familie af enzymer der har et stort potentiale for industriel biokatalyse. Den primære motivation bag dette udsagn er denne families evne til at hydroxylere ikke-aktiverede kulbrinter med høj specificitet ved brugen af molekylært oxygen som oxidationsmiddel. Familien indeholder mere end 26 000 mangfoldige enzymer fra alle livets riger. Udviklingen af effektive industrille processer besværliggøres dog af enzymernes afhængighed af co-faktor og redox partnere samt deres relativt lave aktivitet og stabilitet. I denne afhandling er en ny systematik fremgangsmåde blevet anvendt til at identificere flaskehalse for P450 katalyserede processor for hermed at guide fremtidig forskning og muliggøre hurtigere implementering i industrien. En metodisk fremgangsmåde er blevet udviklet ved at studere videnskabeliglitteratur omhandlende styring på baggrund af økonomiske indikatorer. Den udviklede metode er blevet valideret gennem en række relevante eksempler. Den sidste del af afhandlingen består af en økonomisk evaluering baseret på en procesmodel samt viden opnået gennem eksperimenter, dette inkluderer en sensitivitetsanalyse af de biologiske parametre protein ekspression og total enzymomsætning.

Igennem hele afhandlingen er relevante eksempler blevet udvalgt og studeret. Det første eksempel gjorde brug af et P450 fusionskonstrukt udtrykt i den velkendte vært *Escherichia coli*, hvori enzymet blev udnyttet til ω-hydroxylering af dodekansyre. Dette system repræsenterer et kunstigt fusionskonstrukt udtrykt i en vært uden naturlig forekomst af P450. P450 stabilitet og aktivitet, co-

faktor regenerering af værtscellen, samt substratinhibering blev identificeret som de største begrænsninger i dette eksempel. Substratinhiberingen blev til dels omgået ved at tilføre substrat i fast form. I det andet eksempel blev værten *Bacillus megaterium*, der naturligt udtrykker P450, benyttet til at udtrykke steroidhydroxylasen CYP106A2 for at foretage 15β-hydroxylation af cyproteronacetat. Den katalytiske aktivitet af the overudtrykte CYP106A2 var afhængigt af værtens naturlige redoxpartner. I dette eksempel blev P450 stabilitet også identificeret som en begrænsning, men også produktinhibering viste sig at være begrænsende. Produktinhiberingen blev i dette tilfælde adresseret ved at introducere et modificeret β-cyklodextrin, resulterende i 98 % omdannelse på gram-skala.

Hel-celles P450 katalyserede processer er blevet identificeret som velegnede til produktion af molekyler af høj værdi. Det er blevet vist af de primære begrænsninger er P450 stabilitet og aktivitet, substrat- og produktinhibering samt værts cellens co-faktor regenereringsevne. Ydermere, er det blevet vist af voksende celler, hvor fermentering og biokatalyse udføres i et trin, er det mest økonomisk rentabel alternativ.

Preface

The work presented in this thesis was mainly conducted in the CAPEC-PROCESS research center at the Department of Chemical and Biochemical Engineering, Technical University of Denmark as partial fulfillment of the requirements for a PhD degree in Engineering. The work was performed between February 2012 and January 2015 and was supervised by Professor John M. Woodley and co-supervisor was Associate Professor Ulrich Krühne.

The work has been performed within the P4FIFTY network, funded by the People Programme (Marie Curie Actions) of the European Union's 7th Framework Programme (FP7/2007-2013) under REA Grant Agreement 289217. This network has enabled several collaborations with both European Universities and companies. In particular with Professor Bernhard Hauer and Sandra Notonier at the University of Stuttgart, Germany resulting in Paper II, Professor Rita Bernhardt and Flóra Márta Kiss at Saarland University, Germany resulting in Paper III and with Lonza AG, Visp, Switzerland (Jürgen Riegler and Michael Ringle) resulting in Paper IV.

Kgs. Lyngby

January 2015

Marie Therese Lundemo

Acknowledgements

I acknowledge my supervisors for valuable input to this work. Many thanks to John, not only for your incredible scientific advices and discussions, but also for seeing the person behind the PhD student. I could not have wished for a better supervisor. Thank you Ulrich for being my co-supervisor.

Thank you all former and present members of PROCESS and CAPEC-PROCESS for creating a nice working atmosphere. Thanks to Hema, my team mate in the GMO lab, for nice company during long lab days. I wish you all the best for the future. Andrijana thanks for being a great office mate.

It has been a great pleasure to be a part of the Marie Curie P4FIFTY network. Thank you all fellows and PIs for being a part of the network contributing to nice and fruitful meetings and discussions. Special thanks to Flóra and Sandra for nice collaborations. Michael and Jürgen, thank you for giving me the possibility to work together with you in Visp and showing great hospitality. Also thanks to the other members of the Biotransformation group in Lonza for welcoming me during the external stay. Leo Schmid, thank you for helpful discussions regarding SuperPro designer.

Thanks to my family, mum, dad and Anna, for always supporting and encouraging me, even though you don't really understand what I'm doing.

Last but not least, Pontus, thank you for always being by my side. This thesis would never have existed if it was not for your constant support. Jag älskar dig!

List of papers

This thesis is based on the following papers, referred to in the text by their Roman numerals.

I. LUNDEMO, M.T. and WOODLEY, J.M., 2015. Guidelines for Development and Implementation of Biocatalytic P450 Processes. Appl Microbiol Biotechnol, 99: 2465-2483.

II. LUNDEMO, M.T., NOTONIER, S., STRIEDNER, G., HAUER, B., WOODLEY, J.M., 2015. Process limitations of a whole-cell P450 catalyzed reaction using a CYP153A-CPR fusion construct expressed in *Escherichia coli*, Appl Microbiol Biotechnol, Submitted.

III. KISS, F.M., LUNDEMO, M.T., ZAPP, J., WOODLEY, J.M., BERNHARDT, R., 2015. Process development for the production of 15β-hydroxycyproterone acetate using *Bacillus megaterium* expressing CYP106A2 as whole-cell biocatalyst. Microb Cell Fact, 14: 28.

IV. LUNDEMO, M.T., RINGLE, M., WOODLEY, J.M., Cost assessment of P450 catalyzed whole cell processes, Biotechnol Progr, Manuscript.

List of abbreviations and nomenclature

Abbreviations

α,ω-DCA	α,ω-dicarboxylic acid
ω-OHFA	ω-hydroxylated fatty acid
15β-OH-CPA	15β-hydroxycyproterone acetate
ACN	Acetonitrile
AdR	Adrenodoxin reductase
Adx	Adrenodoxin
B. megaterium	*Bacillus megaterium*
B. subtilis	*Bacillus subtilis*
Bacillus sp.	*Bacillus* species
CD	Cyclodextrin
CIP	Cleaning in place
CPA	Cyproterone acetate
CYP	Cytochrome P450 monooxygenase
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DSP	Down-stream processing
E. coli	*Escherichia coli*
EC number	Enzyme Commision number
EHS	Environmental, health and safety
G6PDH	Glucose-6-phosphate dehydrogenase
HPLC	High-performance liquid chromatography
HP-β-CD	2-hydroxypropyl-β-cyclodextrin
ISPR	*In situ* product removal
ISSS	*In situ* substrate supply
LB	Luria Bertani
LCA	Life cycle assessment
MP	Main product
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NMR	Nuclear magnetic resonance
P. oleovorans	*Pseudomonas oleovorans*
P. putida	*Pseudomonas putida*
P450	Cytochrome P450 monooxygenase
PBR	Packed bed reactor
Pseudomonas sp.	*Pseudomonas* species
SIP	Sterilization in place
SPD	SuperPro Designer
STR	Stirred-tank reactor

Streptomyces sp.	*Streptomyces* species
TB	Terrific Broth
UPLC	Ultra performance liquid chromatography
Y. lipolytica	*Yarrowia lipolytica*
YADH	Yeast alcohol dehydrogenase

Nomenclature	**Description**	**Unit**
	Biocatalyst yield	g/g cdw
	Product concentration	g/L
	Reaction yield	%
	Space-time yield	g/L/h
cdw	Cell dry weight	g
cww	Cell wet weight	g
DCW	Dry cell weight	g
DOT	Dissolved oxygen tension	mol/L
k_{cat}	Turnover number	s^{-1}
k_la	Volumetric mass transfer coefficient	h^{-1}
K_m	Michaelis constant	M
logP	Octanol/water partition coefficient	Dimensionless
Mw	Molecular weight	kDa
OD_{600}	Optical density at 600 nm	Dimensionless
pO_2	Partial oxygen pressure	%
TTN	Total turnover number	Dimensionless
U/g cdw	Specific activity	µmol/min/g cdw
V_{max}	Maximum velocity	kat, nmol product/nmol enzyme/min
vvm	Aeration rate	L/L/min
WCW	Wet cell weight	g

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1 Introduction

1.1 Background

Traditional chemical processes are based on oil as a feedstock and also include harsh reaction conditions in many cases. One way of limiting the consumption of fossil fuels and reducing the emissions of greenhouse gases such as CO_2 is to find alternative process options. More sustainable options gaining ground are bioprocesses, a popular term including both biobased processes referring to use of renewable feedstocks, fermentation processes and biocatalytic processes utilizing a renewable enzymatic catalyst. The terminology applied within this field can easily lead to confusion, bio-based processes are referring to the feedstock but the process itself could be a biological or chemical process. Within the bioprocesses the processes could be divided firstly into fermentation processes producing high molecular weight products such as enzymes or antibodies and secondly biocatalysis where an enzyme is catalyzing a reaction, which is the type of process dealt with in this thesis. It should however be noted that biocatalytic processes do not always use a bio-based feedstock and could also include fermentation. Biocatalytic processes have emerged complementary to or replacing traditional chemical processes. These processes are usually performed in aqueous solutions under mild conditions (close to room temperature, neutral pH, atmospheric pressure) and in this way are generally more environmentally friendly compared to traditional chemical processes.

1.2 Industrial biocatalysis

Industrial biocatalysis (industrial biotechnology or white biotechnology) is the biological alternative to traditional chemical synthesis. Instead of metal catalysts such as Rh, Ru, Pd and Pt, enzymes are applied to catalyze the desired reaction. Enzymes are defined as proteins capable of catalyzing chemical changes in organic compounds. Enzymes are produced via fermentation, where a microorganism is cultivated to produce the enzyme of interest. Commonly, the microorganism is modified to overexpress the enzyme and the enzyme can be produced either intracellular (inside the

cell) or extracellular (enzyme exported outside the cell). In recent years enormous progress in the application of enzymes for industrial chemistry has been witnessed, in particular in the pharmaceutical sector (Wohlgemuth 2010; Woodley 2008; Bornscheuer et al. 2012). Enzymes or rather enzymatic reactions are classified according to the type of reaction performed into 6 different classes assigned an Enzyme Commision (EC) number. For example, potentially important types of enzymes for industry are oxidoreductases (EC class 1) including cytochrome P450 monooxygenases, transferases (EC 2) such as transaminases and hydrolases (EC 3) including lipases. Lipases are active and highly stable enzymes and are applied for e.g. the production of biodiesel, a low value chemical/ fuel, but also in pharmaceutical processes. Transaminases are used for production of chiral amines (high value pharmaceutical intermediates) (Savile et al. 2010). However, one class of enzymes commonly described to have a lot of potential for industrial biocatalysis that remains particularly challenging to implement is the enzyme family known as cytochrome P450 monooxygenases (P450s or CYPs). Cytochrome P450s perform highly interesting chemistry for industry but their biological characteristics as described in section 1.8 hinders the implementation.

The conditions under which enzymatic reactions are performed in nature are usually at low concentrations and controlled by inhibition by the product. This is nature's way of creating highly efficient catalysts being able to regulate cellular reactions and the implication is that the K_M, the Michaelis constant, is very low, describing that the maximum rate of which an enzymatic reaction is being catalyzed is at low substrate concentrations. Under physiological conditions, the enzyme expression is also upregulated or enzymes broken down to control the need of this particular catalytic reaction. These conditions are very different from the demands for industrial application, with high demand on catalyst stability at high concentrations of substrate and product. The reason for these demands are so that it is possible to reach efficient, economical feasible processes. Enzymes can be improved to function better under desired conditions by protein engineering through rational protein design, where new mutants are created by site-directed mutagenesis, or by directed evolution, where large mutant libraries are created by random changes and mutants selected by desired properties (Buchholz et al. 2012). This has also been implemented for P450s aimed at improving not only selectivity and activity (Seifert et al. 2011; Furuya et al. 2012; Malca 2012), but also screening for

P450s performing novel reactions or engineering P450s to perform novel reactions (McIntosh et al. 2014).

1.3 Biocatalyst formulation

Biocatalytic processes starts with a fermentation step, where the biocatalyst is produced. The enzyme of interest is typically overexpressed in a host cell, bacterial or fungal, and depending on the biocatalyst formulation the process structure after the initial fermentation will vary. The biocatalyst can be used as a whole cell, growing or resting, still metabolically active, or go through further formulation steps. These steps typically consist of a centrifugation/filtration step followed by cell disruption (for intracellular enzymes) and purification to the desired purity. The enzyme can then be applied free, soluble, in the crudest form without purification (commonly called crude cell extract), purified or immobilized (by binding to a carrier, entrapment in matrices or by cross-linking (Sheldon 2007)). The biocatalyst can also be purchased in the desired formulation, and then the initial fermentation and biocatalyst processing steps are excluded from the biocatalytic process.

1.4 Economic feasibility

Despite the many advantages of biocatalytic processes compared to chemical processes, the economic feasibility is of major importance and is what will determine if a process finally will be implemented. Although environmental assessments are of increasing importance, the economic assessment is still the key factor that will determine the fate of a process. To guide required biocatalyst improvement and early process development, economic metrics can be applied (Table 1.1). The concept can be implemented at an early stage of process development to guide further development, as an *in silico* engineering approach (Lima-Ramos et al. 2014). The metrics applied in this thesis are reaction yield, final product concentration, space-time yield (also known as volumetric productivity) and biocatalyst yield. The demands on these metrics depend on the area of application (e.g fuel or pharmaceuticals) and market size. The demands on all metrics, individually or combined, reflects different costs of the process. However, the minimum threshold values for each individual metrics must be met to fulfill the demands of an economical feasible process. Reaction yield and final product concentration can be applied to biocatalytic processes regardless of the operating mode and

mainly reflects the cost contribution from down-stream processing (DSP). The difference in cost of the substrate and price of the product gives a first indication of demands on the reaction yield. Space-time yield, or productivity, is mainly used to guide the development of growing cell processes, which together with desired amount of product to be produced, represents the cost of the equipment. For a resting cell process, this is also of importance but can be more easily controlled by the catalyst concentration applied in the process. Biocatalyst yield is applied to resting cell processes and other processes where the biocatalytic reaction is performed independent from the initial fermentation step, and reflects the cost of the biocatalyst production (fermentation and biocatalyst processing). Target values used for an initial analysis of a potential P450 catalyzed whole-cell process are presented in Table 1.1 and used for the analyses performed in Chapter 2.

Table 1.1 Economic metrics to guide early development of biocatalytic processes.

Metric	Mainly applied to	Unit	Target value
Reaction yield	Growing and resting cell processes	%	>90
Final product concentration	Growing and resting cell processes	g/L	20
Space-time yield	Growing cell processes	g/L/h	2
Biocatalyst yield	Resting cell processes	g/g cdw	10

1.5 Cytochrome P450 monooxygenases

Cytochrome P450 monooxygenases are heme containing enzymes found in all kingdoms of life. They are a diverse group of enzymes named after the characteristic peak of the reduced CO-bound complex at 450 nm and include more than 26 000 enzymes (Nelson 2009). This enormous diversity also implies that P450s are of relevance for a broad range of research fields, not least because of their involvement in human drug metabolism (Rendic and Guengerich 2010; Gillam and Hayes 2013; Martinez and Rupashinghe 2013). However, in this thesis, the focus has been on P450s with potential for synthetic chemical production at industrial scale.

1.5.1 Classification and nomenclature

P450s are as mentioned above classified under oxidoreductases, performing oxidation and reduction reactions (EC 1). The classification is further divided into oxygenases (introducing oxygen atoms into their substrates), monooxygenases (introduces one oxygen atom into their substrate), external monooxygenases and finally P450 monooxygenases (Figure 1.1). To cover all P450s, the EC number applied is 1.14.X.Y.

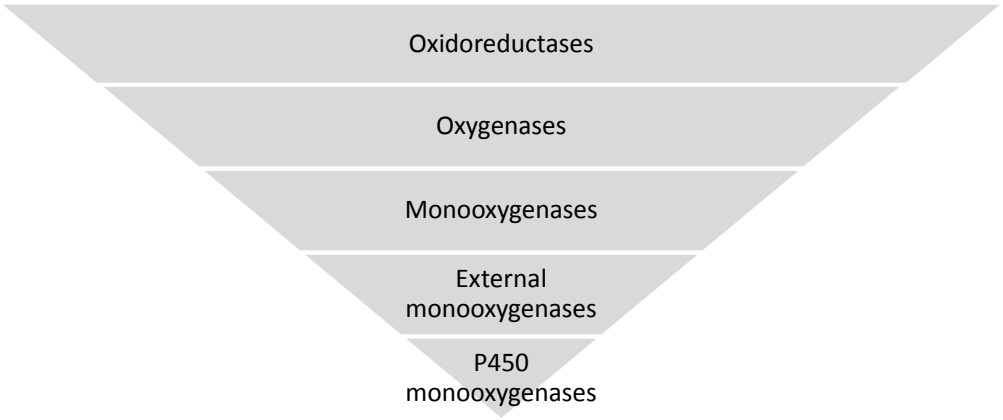


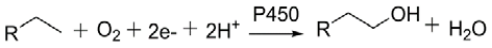
Figure 1.1 Classification of P450s into EC. 1.14.X.Y

The official nomenclature for cytochrome P450 monooxygenases is CYP followed by an Arabic number representing the family and a letter representing the subfamily and a final Arabic number representing the individual gene (Nelson et al. 1993). Generally, the sequence identity within a family is >40 % and within mammalian subfamilies the sequence identity is >55 %.

1.5.2 Chemical reactions catalyzed by P450s

P450s are in general challenging to implement in industry and there are few examples of the oxidation of non-natural substrates. Perhaps surprisingly, given the challenges, the scientific literature reports that cytochrome P450 monooxygenases are potentially amongst the most useful of all enzymes to exploit as industrial biocatalysts since they possess the ability to catalyze the oxidation of non-activated hydrocarbons in a stereoselective manner, including large molecules such as e.g.

steroids, also exemplified in Chapter 4 (De Montellano 2005; Julsing et al. 2008; Schulz et al. 2012). A general reaction scheme is shown in Scheme 1.1 and examples of possible reactions types in Figure 1.2, selected from Sono et al. (1996). The first hydroxylation can also enable further oxidations and other chemical reactions as summarized by Bernhardt and Urlacher (2014). More unusual P450 catalyzed reactions have also been summarized in a recent review (Guengerich and Munro 2013). Interestingly, this selective chemistry is hard to achieve by conventional chemical routes since heterogeneous catalysis and organometallic activation suffer from side reactions and other drawbacks such as high temperature operation (Labinger 2004). In fact, the selectivity of these enzymes far outcompetes their chemical counterparts, but it is their productivity which has been found limiting. The lack of obvious competing chemical processes should, therefore, make the biocatalytic (P450-based) processes economically interesting. However, for products without competitive chemical processes, the market price of the intended products can be a challenge to determine and will be further elaborated on in the discussion in Chapter 6.



Scheme 1.1 General reaction scheme for a P450 catalyzed reaction.

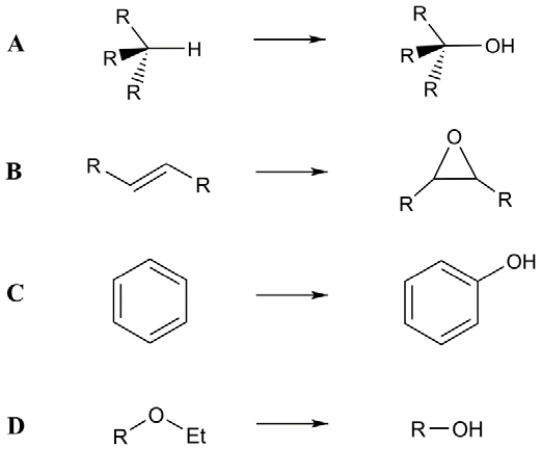


Figure 1.2 Example of reactions performed by P450s. **A**: hydroxylation of hydrocarbons exemplified by activation of sp3 hybridized carbons, **B**: epoxidation of carbon carbon double bonds, **C**: aromatic hydroxylation, **D**: Dealkylation. Reactions selected from Sono et al. (1996) and picture modified from O'Reilly et al. (2011).

1.5.3 Biological characteristics

The main biological characteristics of cytochrome P450 enzymes are the heme prosthetic group, the requirement for a nicotinamide cofactor and the corresponding electron transfer proteins (reductases). P450s are mainly dependent on NADPH, although NADH is also used by some classes. P450s are divided into classes depending on the nature of the redox partner. The catalytically active complex can be a one component system, where the redox partner is fused to the monooxygenase, or two or three components (Figure 1.3). P450s have been divided into 10 classes dependent upon the electron transport chain in a given case (Hannemann et al. 2007) and can be classified into one (class VII, VIII, IX and X), two (class II, V and VI) and three (class I, III and IV) component systems (Urlacher and Girhard 2012). Furthermore, P450s can be soluble or membrane bound proteins, the latter more common among mammalian enzymes.

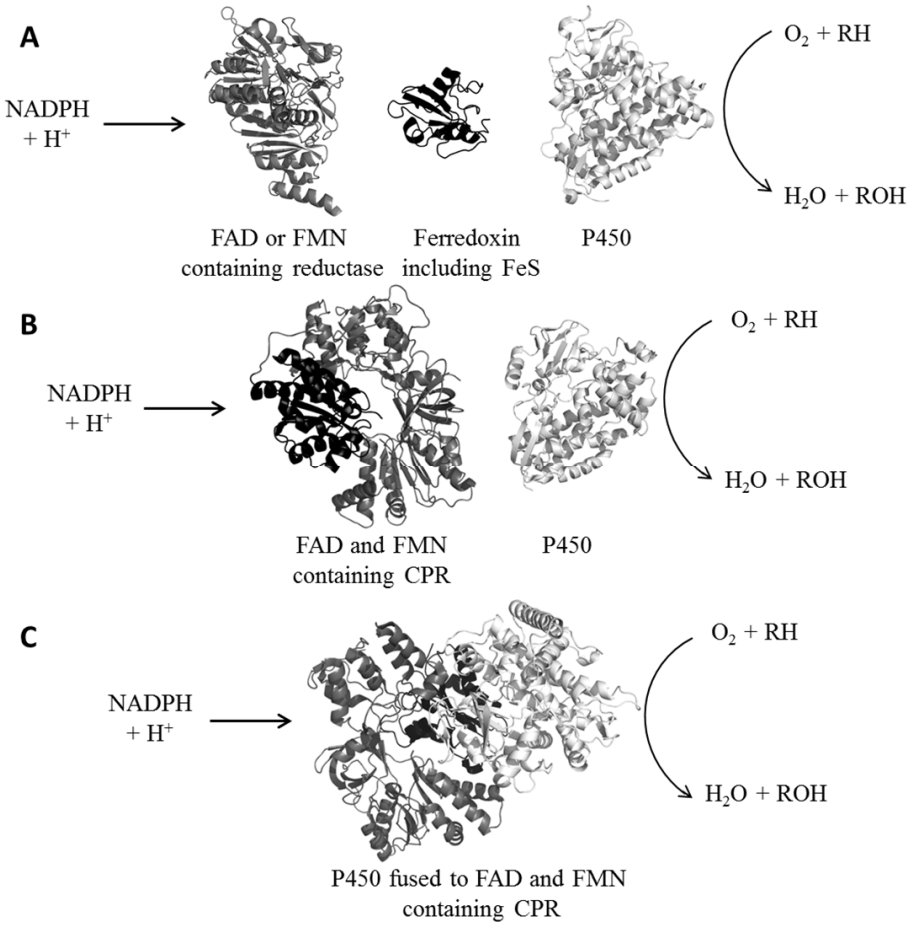


Figure 1.3 Illustration of **A**: three protein systems (e.g. class I), **B**: two protein systems (e.g. class II) and **C**: one protein systems (e.g. class VIII). (Structures based on PDB code 1Q1R, 1PDX, 2ZWT; 1JAO, 1F4U; 4DQK, 1BVY). Picture modified from Urlacher and Girhard (2012).

Additionally to the biological characteristics described above, relatively low activity and stability of P450s makes a whole cell the preferred biocatalyst formulation. Whole-cell systems provide a cofactor regeneration system, possible co-expression of redox partners and a protected environment to enhance the stability of the biocatalyst. Furthermore, a whole-cell biocatalyst is considerably

cheaper than the isolated or immobilized enzyme, if the extra cost for catalyst formulation cannot be justified by catalyst recycle (Tufvesson et al. 2011).

1.6 Scope of the thesis

The main goal of this thesis is to identify and address bottlenecks for whole-cell P450 catalyzed processes to enable industrial implementation. Although P450s perform challenging chemistry from a traditional chemical perspective, hydroxylation of non-activated hydrocarbons, there are many challenges with the industrial application of this class of enzymes. The biological nature of the enzyme is by itself associated with many limitations for this area of application.

1.7 Layout of the thesis

As a start of this thesis, the potential of P450s for application in biocatalytic processes have been summarized with the basis of published literature and demands on an economical feasible process (Chapter 2). The identified limitations have then been experimentally confirmed using two model systems (Chapters 3 and 4). The first one is a P450 (CYP153A) fused to a reductase forming a larger protein and expressed in the most common bacterial host used in research, *Escherichia coli* (Chapter 3). The second model system used consists of an overexpressed P450 (CYP106A2) in another bacterial host, *Bacillus megaterium* (Chapter 4). The gained experiences from primarily the first model system (CYP153A expressed in *E. coli*) were subsequently applied to a third model system and the knowledge was transferred to Lonza Chemie AG (Chapter 5 and Appendix 1). Based on this work a process model was built in the program SuperPro Designer to generate a cost model guiding further development (Chapter 5). The thesis is finally concluded with a general discussion, conclusions and recommendations for future work.

2 Potential of P450 catalyzed processes

The first part of the thesis has been to identify opportunities and limitations for whole-cell P450 based processes. Published literature has been summarized and put in perspective of demands on an economically feasible, industrially suitable P450 catalyzed whole-cell process. This chapter is based in large part on our review paper published in *Applied Microbiology and Biotechnology* (Paper I).

In the last few years alone, several reviews have been published covering many aspects of the cytochrome P450 family and their potential in biotechnology, and the chemical- and pharmaceutical industry (O'Reilly et al. 2011; Urlacher and Girhard 2012; Grogan 2011; Julsing et al. 2008; Jung et al. 2011). The most recent review by Bernhardt and Urlacher (2014) is an excellent summary of the progress made within P450 research for biotechnological applications. Hence, the objective of this chapter is to take this one step further and present an analysis of improvements that can be made to whole-cell cytochrome P450 monooxygenases systems, using both biological and process engineering tools, to enable industrial implementation of economically feasible P450 catalyzed production of chemicals and pharmaceuticals. Important steps will be discussed from a process perspective with the intention of bridging the gap between the extensive molecular biology research within the field of P450s and future process implementation. The intention is to identify areas where more research is needed and to guide efforts aimed at developing a process for this group of catalysts, which has more biological than chemical challenges compared to many other biocatalytic processes. This is done by identifying bottlenecks, divided into catalyst, reaction or process related parameters preventing us from reaching the process targets.

2.1 Toward processes for P450-based systems

2.1.1 Whole-cell processes

In whole-cell processes, the host plays a crucial role in the overall process structure and this has been emphasized in an excellent recent review by Schrewe and co-workers (Schrewe et al. 2013). Exploration of recombinant prokaryotic and eukaryotic expression systems is a key step toward

industrial implementation of such biocatalysts. However, a heterologous recombinant host might not be superior to the natural P450 expression host and high expression levels in the natural host have been suggested to improve the performance. The specific activity of the alkane monooxygenase component AlkB was e.g. reported to be five to six times higher in the natural host *P. oleovorans* compared to *E. coli* (Staijen et al. 2000). Despite the possible overexpression in *E. coli* (2-10-fold), this did not compensate for the lower activity. Indeed those processes which have reached industrial maturity mainly utilize native microorganisms (Liese et al. 2006). There are, however, some exceptions such as the impressive synthetic biology work of an entire pathway, including P450s, leading to production of artemisinic acid, a precursor to the antimalarial artemisinin, by *Saccharomyces cerevisiae* with a product concentration of 25 g/L (Paddon et al. 2013). This work was funded by Bill and Melinda Gates Foundation and the drug is being sold on a non-profit basis. Even though the result is very inspiring, for commercial products in general the development cost has to be accounted for in the overall performance. Another engineered pathway including several P450s from higher eukaryotes with glucose/ethanol as a substrate in *Saccharomyces cerevisiae* was developed for the production of progesterone and later hydrocortisone (Szczebara et al. 2003; Duport et al. 1998). The reported product concentrations are, however, in the mg/L-scale. Metabolic pathway engineering has also been applied in *Escherichia coli* for production of precursors in the synthesis of anticancer drug taxol where a plant P450 is involved in the next step producing taxadien-5α-ol (Ajikumar et al. 2010). However, the P450 catalyzed reaction was shown to be the limiting step in the engineered host and a titer of 60 mg/L was achieved. Besides native hosts such as *Bacillus sp*. (Bleif et al. 2012), *Pseudomonas sp.* (Kuhn et al. 2012), *Streptomyces sp.* (Park et al. 2003) as well as several different yeast strains (*Saccharomyces cerevisiae* (Szczebara et al. 2003), *Schizosaccharomyces pombe* (Drăgan et al. 2011), *Pichia pastoris* (Kolar et al. 2007), *Yarrowia lipolytica* (Nthangeni et al. 2004)) non-natural P450 expressing hosts exemplified by recombinant *E. coli* have been used as expression systems for P450s (Scheps et al. 2011; Zhang et al. 2010). *E. coli* lacks intrinsic P450 genes and heme proteins, and hence might require addition of the heme-precursor (δ-aminolevulinic acid), whereas the yeast strains to varying extent have their own P450 expression systems (as well as an internal membrane

system) simplifying the expression of eukaryotic membrane bound P450s even though this can be achieved also in *E. coli* (Richardson et al. 1995).

In addition to selection of the host cell, the operating mode is also important for the process (as illustrated in Figure 2.1). Two options are possible. In the simpler approach, growing cells (where fermentation and biocatalysis are performed simultaneously) is used. Alternatively, resting cells (where fermentation and biocatalysis can be optimized independently) enables different possibilities for process improvement. Previously, the choice of biocatalyst form from a process perspective was discussed, and it was concluded that decisions need to be made on a case-by-case basis (Woodley 2006; Wohlgemuth and Woodley 2010).

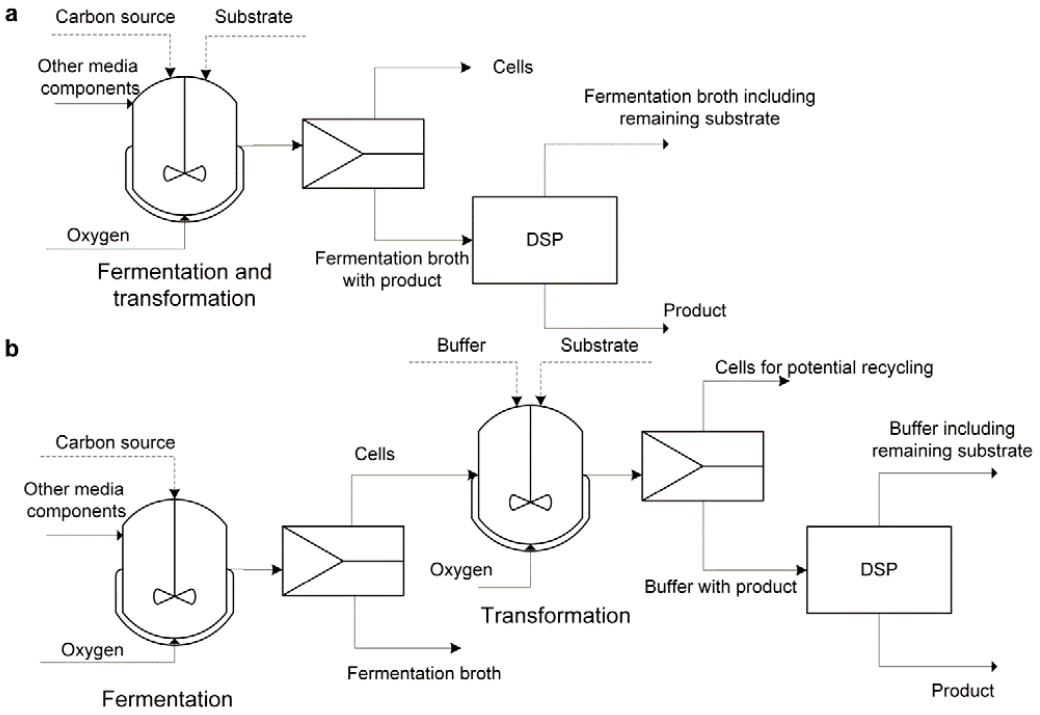


Figure 2.1 a) Fermentation and conversion performed in one step with growing cells. **b)** Fermentation and conversion are separated by centrifugation (or filtration) enabling optimum conditions for the fermentation and conversion and use of the cells in a resting state. The proposed figures assume that the product is present in the media or buffer and not associated with the cells. DSP – Downstream processing.

2.1.2 Economic metrics

The economic potential of a biocatalytic process can be described using metrics (Lima-Ramos et al. 2014; Van Dien 2013). Demands on reaction yield (g product/g substrate) and final product concentration (g/L) can be applied regardless the operating mode. Space-time yield (g product/L/h) is, however, more important to growing cell processes in line with fermentative production of bulk chemicals (Oudshoorn et al. 2010), although with more flexible threshold values due to the higher product value. Besides the final product concentration, important for downstream processing (DSP),

the cost of the equipment required to reach desired amounts of product is an important factor in a one-pot growing cell process. This cost is best translated to demands on space-time yield. For scaling of a process, the space-time yield also plays a role in a resting cell process. For a resting cell processes (and for processes where further biocatalyst processing is used) biocatalyst yield (g product/g catalyst) is applied instead. This metric represents the total amount of product that can be produced by the catalyst, similar to a total turnover number. The biocatalyst yield reflects the cost contribution of the catalyst itself and the target value is influenced by the final cell density and protein expression of the fermentation process. Catalyst recycle is one approach to improve the biocatalyst yield. The requirements for all metrics are dependent upon the product application and market size. The value added by the process is determined by the selling price of the product multiplied by the reaction yield minus the cost of the substrate. Before considering process development, a reaction yield above 90 % is recommended to be able to reach the economic targets. The reason is that the similar properties of substrate and product make DSP difficult. The reaction yield is determined not only by the substrate concentration but also by the specificity of the enzyme. Overoxidation by the P450 of interest or other pathways in the cell as well as oxidation by other P450s will influence the product profile. Byproducts will strongly contribute to more challenging DSP due to very similar properties of many unwanted byproducts compared to the target molecule. Although a reaction yield above 90 % has not been achieved for all whole cell processes summarized later in this chapter, there are examples from a resting (Fujii et al. 2009) and growing cell process (Kleser et al. 2012). Furthermore, it is important to minimize byproduct formation enabling efficient utilization of the whole cell in terms of e.g. cofactor availability (further discussed below). Reasonable target values for product concentration is above 20 g/L and space-time yield around 2 g/L/h (growing cells) and these numbers are also fulfilled in successfully implemented industrial biocatalytic processes (Straathof et al. 2002; Oudshoorn et al. 2010; Van Dien 2013). For a resting cell process, with potential additional biocatalyst processing step and recycle, a target value for the biocatalyst yield for pharmaceuticals or high value chemicals is around 10 g/g cell dry weight (g/g cdw). Each metric reflects demands on different steps of the process, and in the case of hydroxylation reactions by P450s the required reaction yield is expected to influence the DSP cost to a higher extent than in other processes due to very similar properties of the

substrate and product. Typical DSP depends on the product localization, if the product is accumulated inside or attached to the cells or soluble in the media, determining if the cells need to be separated or disrupted. Extraction by organic solvents and further purification is a possible scheme and also enables the DSP to be retrofitted to an existing chemical plant.

In Table 2.1, the typical parameters relevant for whole-cell P450 catalyzed reactions, divided in biocatalyst related parameters, substrate and product related parameters and process related parameters, are shown and what metric they primarily influence. It should be emphasized that the targets defined for each metric need to be fulfilled for an economical viable process. Furthermore some of the parameters are intrinsic to the relevant system and thereby set constraints for the process.

Table 2.1 Typical biocatalytic-, substrate and product- and process related parameters relevant for a whole-cell P450 catalyzed reaction and what metric they primarily influence. [a] The toxicity to a cell is considered to be irreversible and thereby primarily influence biocatalyst yield and final product concentration whereas [b] inhibition to the enzyme can be both reversible and irreversable and can thereby also affect the space-time yield. Units for the metrics: [c] total amount of product produced per gram cell dry weight (g/g cdw), [d] final product concentration in the reaction media (g/L), [e] amount of product per amount of substrate (g/g), [f] amount of product produced per volume of reaction media per time (g/L/h).

PARAMETER			METRIC			
			Biocatalyst yield[c] (g/g cdw)	Final product concentration[d] (g/L)	Reaction yield[e] (g/g)	Space-time yield[f] (g/L/h)
Biocatalyst related parameters	Specific activity (U/g cdw)	Fermentation cell density (g cdw/L)				X
		Enzyme expression (nmol/g cdw)	X			
		Enzyme activity (s^{-1})				X
		Cofactor regeneration (µmol/min/g cdw)				X
		Electron transport (s^{-1})				X
		Coupling efficiency (%)			X	
	Stability	Cell (h)	X	X		
		Monooxygenase (TTN or h)	X	X		
		Reductase (TTN or h)	X	X		
		Cofactor (TTN or h)	X	X		
Substrate and product related parameters		Transport across membrane (µmol/min/g cdw)				X
		Solubility (g/L)		X		
		Toxicity[a] (g/L) or (g/g cdw)	X	X		
		Inhibition[b] (g/L) or (g/mol P450)	X	X		X
Process related parameters		Oxygen (mol/L/h)				X
		Transport between phases (g/L/h)				X

Economic assessments regarding catalyst production for application in processes where the biocatalytic reaction is separated from the fermentation step have been made and these assumptions can be translated to biocatalyst yield targets of at least 10 g product/g dry cell weight for pharmaceuticals and significantly higher numbers for lower value bulk chemicals (Tufvesson et al.

2011). This was calculated on a base case with a protein expression of 6.25 g/L and a cell titer of 50 g cdw/L. To put these numbers into perspective the highest reported expression of functional P450s to our knowledge is 1.5 g/L with a cell titer of 13 g cdw/L (Pflug et al. 2007). To reach the target product concentration of 20 g/L, a specific activity of 7 µmol substrate converted per gram cell dry weight per minute (U/g cdw) would be required. These calculations are based on numbers from Pflug et al. (2007) (13 g cdw/L, a molecular weight of the substrate, p-nitrophenoxydodecanoic acid, of 138 g/mol and product of 154 g/mol) for a 24 h process with constant activity. Doing similar calculation for a growing cell process based on a space-time yield requirement of 2 g/L/h, the required specific activity would be 17 U/g cdw. Specific activities in this range have been reported and are not unrealistic. For a resting cell process, without cell recycling, 10 g/g cdw corresponds to 45 U/g cdw, indicating that cell recycle might be necessary to cover the extra cost contribution from the biocatalyst processing step (filtration or centrifugation and washing in case of whole cells). However, to assume a constant activity over 24 h is not really realistic in the case of P450s. Several challenges to achieve the required targets for each metric can be expected and in the following sections this will be discussed along with possible solutions, already summarized in Table 2.2. Table 2.1 and Table 2.2 have the same basis to simplify the correlation between parameters influencing the reaction and tools to improve the system in order to reach a target metric in the end.

Table 2.2 Summarized challenges for whole-cell P450 catalyzed processes with proposed ways of identification and solutions.

PARAMETER			How to identify bottleneck?	How to overcome bottleneck?	Examplified by
		Fermentation cell density (g cdw/L)	Economic calculations and cdw measurements	Fermentation optimization	(Zhang et al. 2010; Tufvesson et al. 2011; Pflug et al. 2007)
Biocatalyst related parameters	Specific activity (U/g cdw)	Enzyme expression (nmol/g cdw)	Economic calculations, cdw measurements and SDS-PAGE or CO differential spectra	Fermentation optimization	(Tufvesson et al. 2011; Pflug et al. 2007; Zhang et al. 2010)
		Enzyme activity (s^{-1})	*In vitro* assays	Protein engineering	(Whitehouse et al. 2012)
		Cofactor regeneration (µmol/min/g cdw)	Addition of external cofactor	Change mode of operation Metabolic engineering, Protein engineering, Coexpression of eg. Dehydrogenase, Direct supply of electrons	(Fasan et al. 2011; Lee et al. 2013; Schewe et al. 2008; Siriphongphaew et al. 2012; Mouri et al. 2006; Hollmann et al. 2006)
		Electron transport (s^{-1})		Engineering of redox partners Design of fusion construct	(Bell et al. 2012; Hollmann et al. 2006; Zehentgruber et al. 2010a; Ewen et al. 2012; Scheps et al. 2013; Bordeaux et al. 2011; Robin et al. 2009; Hakki et al. 2008)
		Coupling efficiency (%)	Compare consumption of cofactor and formation of product	Protein engineering	(O'Reilly et al. 2011; Fasan et al. 2007)
	Stability	Cell (h)	Follow viable cells over time	Host cell selection, Immobilization	(Liu et al. 1998)
		Monooxygenase (TTN, h)	CO differential spectra	Protein engineering, Immobilization Addition of stabilizing agents	(Cirino and Arnold 2003; Salazar et al. 2003; Seng Wong et al. 2004; Maurer et al. 2005; Maurer et al. 2003; Weber et al. 2010; Lamb et al. 1998; Hirakawa and Nagamune 2010)
		Reductase (TTN, h)		Protein engineering or alternative partner	(Munro et al. 2007a; Eiben et al. 2007)
		Cofactor (TTN, h)		Change mode of operation Change reaction media	
Substrate and product related parameters		Transport across membrane (µmol/min/g cdw)	Reactions with crude extract	Permeabilization of membrane Host cell selection Coexpression of transporter	(Zehentgruber et al. 2010b; Bleif et al. 2012; Julsing et al. 2012; Schneider et al. 1998; Cornelissen et al. 2013)
		Solubility (g/L)	Data from literature	Water miscible or immiscible solvents	(Panke et al. 2000; Braun et al. 2012)
		Toxicity (g/L), (g/g cdw) Inhibition (g/L), (g/mol P450)	Reactions at varying conc. of substrate and product	Substrate feeding, ISSS and ISPR by resins or second phase, Host cell selection	(Siriphongphaew et al. 2012; Zehentgruber et al. 2010a; Schewe et al. 2009)
Process related parameters		Oxygen (mol/L/h)	kla measurements or experiments with increasing catalyst concentration	Increase oxygen content in air, mass flow or agitation Alternative reactor configuration	(Hilker et al. 2004)
		Transport between phases (g/L/h)	Lewis cell experiment	Addition of surfactants/cosolvents	(Ryan and Clark 2008)

In order to identify and summarize where the P450 research stands in relation to the defined guidelines above, selected growing and resting whole-cell processes have been summarized and are presented in Figure 2.2. The best performance among the processes, in relation to the presented targets, is found using an unspecified P450 in a growing natural P450 expressing mutant strain of *Candida tropicalis*. This uncharacterized P450 is converting n-tridecane to the corresponding dicarboxylic acid in a batch process where fermentation and conversion were separated into two steps, although in the same fermenter (Liu et al. 2004). A step-wise addition of alkane and optimized pH profile, by gradual increase during the production phase, were applied to the process. The process performance is in the same order of magnitude regarding space-time yield and product concentration as the minimum guideline for pharmaceutical processes, while other processes are almost two orders of magnitude behind. As can be seen in Figure 2.2, the resting cell processes are further away from the target, expressed as biocatalyst yield, compared to the growing cells where the target is represented as space-time yield. The main reason for this is that the catalyst is rarely reused in P450 catalyzed processes, which would be required to cover the extra cost arising from the additional biocatalyst processing step (centrifugation or filtration). In lab processes cell recycle is scarcely considered and as mentioned, in industrially implemented processes, growing cells has been the preferred operating mode.

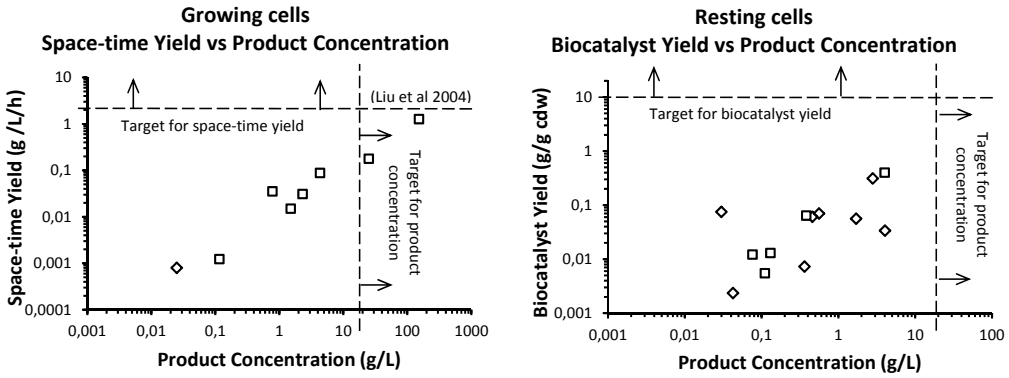


Figure 2.2 Left: Published growing P450 whole cell processes presented as space-time yield vs product concentrations. **Right:** Resting P450 whole-cell processes presented as biocatalyst yield vs product concentration. □ represents reactions performed in bioreactors and ◊ represents shake flask or test tube processes. (Growing cells in this figure defined as processes performed in one pot and space-time yields calculated on entire process time.)

A whole-cell system is a complex machinery and is dependent on the environment, e.g. the availability of cofactor for the desired biocatalytic reaction and the oxygen consumption by the resting cell strongly depends on the state of the cell. Therefore, it is hard to generalize and immediately present required parameters that need to be fulfilled. To be able to make reasonable calculations and guide the catalyst development in the field, some assumptions need to be made to be able to convert e.g. specific activity (U/g cdw) to a space-time yield expressed in g/L/h. Furthermore, in order to correlate activities achieved in short enzymatic assays (e.g. using p-nitrophenoxydodecanoic acid) to reactions with a different substrate in whole-cell systems is not straightforward and depends on several parameters. In the case of α-pinene oxyfunctionalization by a BM3 quintiple mutant, the *in vitro* assay above showed a k_{cat} of 0.7 s^{-1} and the corresponding value for the non-natural substrate α-pinene in a whole-cell with heterologous cofactor regeneration was 0.17, representing a fourfold difference in favor of the *in vitro* assay (Schewe et al. 2008). On the other hand, a more accurate comparison using the same substrate *in vitro* and *in vivo*, shows the opposite trend meaning higher specific activities for the *in vivo* systems (2.9 fold for (S)-limonene hydroxylation by CYP153A6 and 3.9 fold for epoxidation

of 1-hexene by CYP102A1 F87V) indicating that the intact cell provides a better environment for the enzyme (Cornelissen et al. 2011; Siriphongphaew et al. 2012).

2.2 Improving specific activity to give increased space-time yield (g/L/h) or biocatalyst yield (g/g biocatalyst)

The specific activity, expressed as U/g cdw, is affecting the space-time yield (g/L/h) for a growing cell process and the biocatalyst yield (g/g cdw) for a resting cell process. There are two main factors that contribute to the specific activity: enzyme expression (mol P450/g cdw) and enzyme activity, k_{cat} (s^{-1}, turnover per active site and second). However, an increased enzyme activity *in vitro* does not necessarily translate directly to corresponding activity *in vivo* due to the many factors influencing a P450 catalyzed reaction such as cofactor supply, electron transport and proximity and stability of the catalytic components.

2.2.1 Fermentation cell density, enzyme expression and host cell physiology

The fermentation performance is, as discussed earlier, of key importance for the economy of the process, especially in the case of resting cells. To lower the cost contribution of the catalyst it is of special importance to reach reasonable cell densities in the fermentation process. When the guideline for biocatalyst yield (g/g cdw) was calculated a base case density of 50 g cdw/L was assumed which is reasonable for a fed-batch fermentation process (Tufvesson et al. 2011). Overexpression of proteins constitutes a burden to the host cell and for P450 expression, heme depletion has been shown to cause a major stress (Michener et al. 2012). In a reasonably successful fermentation process, an overexpressed protein content of 12.5 % of the cell dry weight can be assumed (Tufvesson et al. 2011) and when expressing CYP102A1 in *E. coli* similar values (11 %) were reported (Pflug et al. 2007). Dependent on the requirement of overexpression of electron transfer proteins in a given case, this gives different potential for expression of the P450 itself and might limit the total potential activity of the cell (specific activity). This has been exemplified by Schewe and coworkers when co-expressing glucose facilitator and dehydrogenase and achieving comparable product concentrations with half the P450 expression (Schewe et al. 2009).

Cell physiology has in several cases been shown to have a crucial impact on the reaction performance. By changing the host strain from *P. putida* GPo12 to *P. putida* KT2440 and the carbon source from octane to citrate a six-fold improved productivity (g/L/h) was achieved in the hydroxylation of (S)-limonene to (S)-perillyl alcohol (Cornelissen et al. 2011). Further work on the same system showed the importance of host background for the formation of byproducts which were decreased from 26 to 8 % by changing host again to *E. coli* W3110 (Cornelissen et al. 2013). Metabolic engineering of the host cell can also influence the performance of the whole-cell system as indicated by an improved product per glucose yield from 0.5 to 1.71 (mol/mol), as a result of more efficient utilization of the cofactor and increased specific activity from 9 U/g cdw to 26 U/g cdw in a resting cell biocatalytic conversion of propane to propanol (Fasan et al. 2011). In a growing NADPH dependent styrene epoxidation on the other hand, the cofactor availability limited the reaction above 21 U/g cdw, probably due to increased consumption of glucose by stress induced processes and NADPH being used inefficiently by the enzyme (Bühler et al. 2008) suggesting metabolic engineering to increase redox biocatalysis. This supports the fact that synthesis and presence of active NADPH decrease growth yields, reduce growth rate and metabolic activity (Bühler et al. 2006).

Direct comparisons of growing and resting processes are not straight forward. First, the basis of the comparison needs to be determined, e.g. the cost driving metric in a specific case needs to be identified. Second, the catalyst concentration in a growing cell process will vary and in a resting cell process the chosen catalyst concentration will influence space-time yield and final product concentration and is an important factor in the comparison. Not many examples can be found in literature where direct comparisons based on process performance have been done and the operating mode has a big influence on process design and should be investigated further. Despite this, in an attempt to compare the two operating modes, *E. coli* overexpressing NADPH dependent cyclohexanone monooxygenase for production of caprolactone using a resting cell process showed 20-fold higher space-time yields (0.79 g/L/h over 10 h) compared to growing cells, although it could be explained by different cell concentrations and the specific activity of the growing cells were higher than the resting cells (Walton and Stewart 2002; Walton and Stewart 2004). The main limitation was shown to be stability of the monooxygenase and substrate transport across the cell membrane. It was

also shown that glucose-fed non-growing cells were not limited by cofactor regeneration (intracellular NADPH concentration after 12 h reaction was 180 µM). On the other hand, *E. coli* W3110 growing in the presence of yeast extract were shown to be more resistant to n-octane compared to resting cells without yeast extract and this lead to a five-fold increase of the final product concentration (Favre-Bulle and Witholt 1992). It has also been suggested that resting cells are more sensitive to product inhibition compared to growing cells in styrene oxidation (Julsing et al. 2012).

2.2.2 Enzyme activity

The highest activity reported to our knowledge is 363 s^{-1} reached by a one component CYP102A1 mutant (Eiben et al. 2006). However, this number is outstanding and reported activities above 1 s^{-1} are scarce with the exception of BM3 and P450cam systems, which are the most explored P450s, and a few other one component systems (P450nor and reductase independent eukaryotic systems)(search on BRENDA 2012-08) (Scheer et al. 2011). In order to reach the lowest target for pharmaceuticals, specified above, of 10 g product/ g dry cell weight, assuming an expression of 11 % enzyme per g cdw and a cell density of 13 g/L, a molecular weight of the enzyme of 118 kDa and a molecular mass of the product of 154 g/mol (numbers taken from Pflug et al. (2007)), the required turnover number for a process lasting 24 h (without cell recycling) would be less than 1 s^{-1}. This implies that the activities reached for most systems are in the range of activities required for a successful industrial process and specially the P450 systems in four families (CYP102, CYP101 and P450nor and independent eukaryotic systems). The enzyme activity can furthermore be improved by directed evolution and site directed mutagenesis and the extensive work done with the most explored P450, P450 BM3, has been summarized up to June 2011 in a review by Whitehouse and colleagues (Whitehouse et al. 2012). In general, these techniques have evolved extensively in recent years and a 10-fold (or even 100-fold) improvement of the enzyme activity is reasonable to achieve (Malca 2012; Lewis and Arnold 2009). To achieve the same improved performance of the enzyme in a whole-cell system as achieved *in vitro*, the enzyme activity would have to be the limiting factor. However, this is, according to calculations above, not the case as long as the assumed stability is fulfilled and, therefore, a direct translation of rates between *in vitro* and *in vivo* is only valid when no other limitations are present.

2.2.3 Cofactor regeneration

In theory, cofactor regeneration for redox catalysis of metabolically active cells has been calculated to be in the range of 500-1000 U/g cdw (Duetz et al. 2001; Meyer et al. 2006; Blank et al. 2008). However, in practice, maximum specific activities for oxygenases (although not P450 monooxygenases) achieved in whole-cells are around 200 U/g cdw (Bühler and Schmid 2004) and the cofactor regeneration has in several cases been shown to limit the activity. Cellular processes such as increased maintenance demand of the cell under biocatalytic conditions as well as byproduct formation and uncoupling (discussed in the following section) can be part of the explanation why the theoretical value has not been reached. Furthermore, growing cells tend to utilize the cofactors for better growth rather than the desired biocatalytic reaction implying that resting cells would be the better operating mode from this point of view. In order to avoid the need for addition of the required cofactor in stoichiometric concentrations, which would make any process uneconomic, several regeneration approaches have been developed. Heterologous coexpression of e.g. glucose dehydrogenase (NADPH), improving the specific activity from 0.39 to 3.59 U/g cdw and 0.96 to 1.47 U/g cdw respectively (Schewe et al. 2008; Siriphongphaew et al. 2012), or glycerol dehydrogenase (NADH) (Mouri et al. 2006) has been used in several cases to improve the cofactor regeneration. Another approach is to couple permeabilized cells, expressing the enzymes of interest, done in the case with NADPH dependent ketoreductase and glucose dehydrogenase used for the production in the gram per liter scale with specific regeneration activities of 61 U/g cdw (Zhang et al. 2009). Recent metabolic engineering approaches for better NADPH regeneration during glycolysis have been reviewed by Lee and co-workers, in the best cases showing a 6-fold improvement of catalytic performance (final product concentration or specific activity) in engineered *E. coli* (Lee et al. 2013). Another approach to tackle the dependence of the more expensive and less stable cofactor NADPH is to change the cofactor specificity to NADH. This was done by site directed mutagenesis for hydroxylation of myristic acid by CYP102A1 (free enzyme with cofactor regeneration by formate dehydrogenase) yielding a total turnover number of 30 000 and a space-time yield of 153 mg/L/h (Maurer et al. 2005).

Aside from regeneration of the cofactor by the host cell and heterologous enzymatic regeneration options, non-enzymatic options to regenerate cofactors and direct regeneration of the monooxygenase active site have been reported and reviewed (Hollmann et al. 2006). NADPH has e.g. been substituted by zinc dust and cobalt(III)sepulchrate, reaching 20 % of the rate achieved using NADPH in vitro for hydroxylation of p-nitrophenoxydodecanoic acid (Schwaneberg et al. 2000). In the case of P450s an alternative mechanism for the reaction is the use of the peroxide shunt pathway as a source of both electrons and oxygen, which was exemplified by hydroxylation of naphthalene by a P450cam mutant showing an 20-fold improvement in activity over the wild-type enzyme (Joo et al. 1999). Cumene hydroperoxide driven dextromethorphan demethylation by CYP2D6 resulted in 210 % of the product concentration achieved by the natural cofactors (NADPH/CPR) after 1 h *in vitro* assay, however, with decreased stability but comparable total turnover numbers (Chefson et al. 2006). The field of peroxide driven P450 catalysis is extensively covered with patents by Arnold and co-workers (Cirino and Arnold 2008).

2.2.4 Electron transport and coupling efficiency

The electron transfer from the cofactor to the heme active site is in many cases the rate limiting step and thereby has been the target for biocatalytic improvement. This has also been suggested to be the reason for observing equal activity in an overexpressed engineered system as in the native host with a lower enzyme concentration (Duetz et al. 2001). Optimization of the flux of electrons is crucial to utilize the full capacity of the biocatalyst to the targeted reaction and the importance has also been emphasized in the review by Bernhardt and Urlacher (2014). Approaches with fusion constructs have been published and besides constructs with the reductase domain of BM3, the reductase domain of CYP116B2 (P450RhF) is a promising alternative (Bordeaux et al. 2011; Robin et al. 2009). This field with a lot of potential for the application of P450s has recently been reviewed (Sadeghi and Gilardi 2013). The length and also structure of the linker within the fusion constructs have been shown to be important for electron transport (Munro et al. 2007b; Robin et al. 2009). Activity can also be reconstituted with more component systems, especially for eukaryotic systems with unknown redox partners, even though the redox partners are commonly known for eukaryotic systems in contrast to prokaryotic ones. The choice of heterologous redox partners is firstly determined by the class of P450

but has proven not to be that straight forward and optimization of the electron transfer has been shown to be beneficial e.g. by a modified ferredoxin (Bell et al. 2012). Putidaredoxin reductase with putidaredoxin (Kim and Ortiz de Montellano 2009) and adrenodoxin reductase with adrenodoxin (Zehentgruber et al. 2010a; Hollmann et al. 2006; Ewen et al. 2012; Hakki et al. 2008) have been the most commonly used redox partners for heterologous expression. Non-enzymatic transfer of electrons to the heme-active site has also been explored, including direct chemical reduction or electrochemical reduction (Hlavica 2009) as well as light driven catalysis exemplified by hydroxylation of lauric acid by P450 BM3 (Tran et al. 2013).

The catalytic efficiency is amongst others affected by uncoupling, when reducing equivalents are lost to byproduct formation without substrate oxidation (O'Reilly et al. 2011). Uncoupling is affected by the physical position of the electron transporting chain (i.e. can be improved by the design of fusion constructs) but especially by the binding of the substrate in the active site and is therefore a particular challenge for efficient catalysis of non-natural substrates. A systematic domain-based directed evolution strategy was used to engineer P450 BM3 towards the non-natural substrate propane (Fasan et al. 2007). This resulted in total turnover number of 45800 and increased coupling efficiency from 17.4 to 98.2% *in vitro*.

2.3 Improving catalyst stability for increased biocatalyst yield (g/g cdw) and final product concentration (g/L)

As already mentioned, a sufficient stability over 24 h is assumed in calculations setting a threshold for enzyme activity. If the stability would only allow for an 8 h reaction, the demand on all rates would be three times higher. This forms thereby a prerequisite and is of major importance. In addition to the stability of the host cell, the stability of the monooxygenase and also the corresponding electron transport proteins are critical to achieve the defined process targets. As stated above, a supplement of cofactors gives severe economic consequences for a scaled process and the cofactor regeneration is one of the motivations for a whole-cell process. The native cofactor regeneration system is, however, not adapted to the activity of an overexpressed enzyme and, besides the regeneration, the stability of cofactors can thereby become a limitation in systems with high turnover numbers and

stable enzymes. Total turnover numbers of up to 1300 for NAD(P)+ and 300 for NAD+ have been reached in an in vitro P450 BM3 biphasic system using formate dehydrogenase (Maurer et al. 2005). In the case of ketoreductase with a glucose dehydrogenase cofactor regeneration approach the overall turnover number for NADPH recycling reached 3400 with a specific activity of 61 U/g cdw (Zhang et al. 2009).

When uncoupling occurs, hydrogen peroxide is produced decreasing the stability of the enzyme by heme degradation (Cirino and Arnold 2003). The stability of P450 BM3 towards hydrogen peroxide has been explored by Arnold and co-workers and the total turnover number was increased 11-fold for a hydrogen peroxide (5 mM) driven P450 BM3 heme domain by protein engineering although the enzyme was inactivated within 5 min in the presence of 10 mM hydrogen peroxide and is far from industrially suitable (Cirino and Arnold 2003). Six additional rounds of directed evolution of this enzyme resulted in a 250 times longer half-life at 57.5 °C compared to the holoenzyme of the wildtype with 50 % remaining activity of the peroxide driven parent (Salazar et al. 2003). The reductase domain of CYP102A1 is known to be less stable than the monooxygenase domain (Munro et al. 2007a). When the reductase domain of CYP102A1 was replaced with the more stable reductase domain of CYP102A3 the stability was improved (10-fold longer half-life at 50 °C) even though the activity was decreased (Eiben et al. 2007).

When designing a whole-cell biocatalytic process with the knowledge that solvents most probably will be used, this should be considered when choosing the host and screening for desired enzymatic characteristics. In general, Gram-negative bacteria are known to be more solvent tolerant than their Gram-positive counterparts. Variations in solvent tolerance can be explained by different membrane composition and efflux pumps and varies not only between different bacterial species but can also vary between different strains of the same species (Ramos et al. 2002).

The stability of P450 BM3 in the presence of co-solvents added to increase the solubility of the substrates has been increased by directed evolution yielding mutants with 10-fold and 6-fold increased specific activities compared to the parent mutant F87A in the presence of 2 % THF and 25 % DMSO respectively (Seng Wong et al. 2004). The improved tolerance was also seen for other co-

solvents (acetone, acetonitrile, DMF and ethanol). P450 BM3 mutants have also been constructed by site-directed mutagenesis for the hydroxylation of cyclohexane in a biphasic system containing 50 % cyclohexane as substrate and organic phase and 50 % aqueous buffer (Maurer et al. 2005). The best double mutant was shown to be active for 100 h when stabilized by catalase and BSA with a total turnover number of 12 850. Similarly, octane (used as substrate and second phase) and myristic acid in dodecane showed total turnover numbers of 2 200 and 3 300 demonstrating applicability of biphasic reaction systems.

Immobilization as a means to increase the stability of P450s has also been explored although to be implemented in industry, this requires improved biocatalyst yield to cover the additional cost added to the process. Mainly purified enzymes have been immobilized and in this case cofactor regeneration has to be addressed. Sol-gel matrix showed to be the best amongst several matrices tested when immobilizing BM3 mutants with immobilized formate dehydrogenase for cofactor regeneration (Maurer et al. 2003). The storage stability was improved measured as the half-life at 25 °C from 5 days (with glycerol) to 29 days. Reusability of the catalyst was also shown. The immobilization of the heme domain of BM3 on mesopourous molecular sieves using hydrogen peroxide as electron and oxygen source was also investigated by Weber and co-workers (Weber et al. 2010). However, the activity towards n-octane was only twice as high compared to the free enzyme and still quite low reaching in total 62 nmol product per mg P450. A fusion protein between the plant CYP71B1 and its reductase showed 10-fold improved activity compared to the free enzyme when immobilized in colloidal liquid aphrons (CLA) (Lamb et al. 1998). Another immobilization approach, taking the importance of proximity of the electron transfer chain for efficient electron transfer into close consideration, called PUPPET where P450cam, putidaredoxin and putidaredoxin reductase where fused to monomers of proliferating cell nuclear antigen (PCNA) forming a trimeric ring structure (Hirakawa and Nagamune 2010). This lead to an increase in initial activity by two orders of magnitude measured by O_2 consumption and NADH consumption compared to the a mixture of the free enzymes leading to a specific activity of 500 min^{-1}.

Cell free extracts of *E. coli* expressing CYP105D1 immobilized on the anion exchange resin DE52 only showed activities of one third to half of the free enzyme but showed activity up to 36 h using formate dehydrogenase for cofactor regeneration (Taylor et al. 2000). Flocculent yeast *Saccharomyces diasticus* expressing a fused P450 enzyme was immobilized within reticulated polyurethane foam biomass particles in a fluidized bed reactor enabling production in 5 batch cycles, each lasting 8 h with higher final product concentration and production rate than freely suspended cells (Liu et al. 1998).

Even though the stability is shown to be increased by protein engineering and immobilization, from a process perspective, it is important that the stability under process relevant conditions is considered (agitation, temperature, concentration of reactants etc.).

2.4 Improving substrate and product related parameters

The biocatalyst can be changed and modified in several ways as discussed above. However, the substrate and product are generally fixed and challenges associated with the reactant(s) and/or product(s) needs to be circumvented by other means. Typical substrates and products of P450 catalyzed reactions have poor water solubility which is a challenge when the whole-cell catalyst is present in the aqueous phase. The transport of the poorly water soluble substrate can limit the space-time yield (g/L/h) if it is lower than the reaction rate and also influences the product concentration (g/L) achievable. Inhibition and toxicity can limit the final product concentration (g/L) and biocatalyst yield (g/g cdw) achievable. Volatility of the substrate or product could potentially limit the reaction yield, especially considering the supply of gaseous oxygen. This is, however, not considered to be a challenge for high value molecules relevant for P450 catalyzed processes due to the large molecular weight of substrates and products and thereby in general also low volatility.

2.4.1 Transport limitations across the cell membrane

Dependent upon the physical properties of the substrate, transport across the cell membrane has in some cases been shown to limit the reaction. Three different mechanisms of hydrocarbon uptake by bacteria have been described in the scientific literature: uptake from aqueous phase, uptake of solubilized substrate in an apolar solvent and from direct contact with organic droplets (Schneider et al. 1998). The membrane needs to allow transport of desired compounds but still be intact enough

not to affect the viability of the cell. In general whole-cell biocatalysis is 1-2 orders of magnitude slower than isolated enzymes due to transport limitations across the cell walls and membranes (Chen 2007). Different permeabilization methods (physical, chemical and molecular engineering approaches) can be applied depending on the microorganism (eukaryotic or prokaryotic, Gram-positive or Gram-negative) and characteristics of your compound (hydrophilic or hydrophobic) and have been reviewed previously (Chen 2007). Zehentgruber and co-workers demonstrated that for steroid transport across the cell membrane in fission yeast permeabilization by Tween 80 increased the activity by 50 % but hexadodecyl trimethyl ammonium bromide (CTAB) extinguished the activity instead, probably due to toxicity to the biocatalyst (Zehentgruber et al. 2010b). Microbial engineering strategies to improve the uptake of hydrophobic substrates by bacteria was applied in the case of dodecanoic acid methyl ester oxygenation in *E. coli* W3110 and a 28-fold increase of maximal specific activity was achieved by co-expression of the AlkL membrane protein (Julsing et al. 2012). Application of a biphasic system further increased the activity (62-fold). However, the expression of AlkL decreased the stability of the cell. The same approach has been taken for alkanes (n-dodecane) in *E. coli* GEC137 (Grant et al. 2011). The examples above are applied to the alkane monooxygeanse system from *Pseudomonas putida* GPo1 and not a P450. Regarding P450s, the fatty acid transport (pentadecanoic acid) was improved in resting *E. coli* K27 by expression of the same gene encoded on pGEc47 from *Pseudomonas oleovorans* from 0.7 U/g cdw to 1.3 U/g cdw (Schneider et al. 1998). The productivity was further improved threefold by the use of cell free extract, although NADPH was added in this case. The AlkL transport protein was also applied in the well-studied limonene hydroxylation by CYP153A6 expressed in *E. coli* W3110 resulting in an increased hydroxylation rate from 7.1 to 16.6 U/g cdw (Cornelissen et al. 2013).

Change of the heterologous expression host can also improve the substrate transport. Utilization of *B. megaterium* MS941 instead of *E. coli* avoided the inability of *E. coli* to transport acids with terpene structure (Bleif et al. 2012). Similarly, hydroxylation of testosterone and diclofenac by human P450s CYP3A4 and CYP2C9 respectively expressed in *E. coli* showed higher specific activity in the form of isolated membranes compared to whole cells indicating transport limitation (Vail et al. 2005). Another option to circumvent the transport limitation across the cell membrane is to express the enzyme by

surface display. CYP106A2 from *Bacillus megaterium* (Schumacher et al. 2012) and human CYP3A4 (Schumacher and Jose 2012) was expressed on the outer membrane of *E. coli* and showed hydroxylation of deoxycorticosterone and testosterone, respectively. It should, however, be noted that the advantages with using the whole-cell system were lost and cofactor and electron transporting proteins need to be added externally. The transport limitations can also imply that the product is accumulated inside the cell (Shimizu et al. 2003) and needs to be recovered through cell lysis, thereby ruling out cell recycle.

2.4.2 Solubility of reactants

Many substrates for reactions catalyzed by P450 have limited water solubility. For example, medium chain alkanes (C5-C16) are water soluble up to around 40 mg/L and likewise fatty acids with the same chain length are soluble in the range of 7 mg/L to 19 g/L. In these cases the transport of the substrate to the water phase containing the catalyst requires an immiscible or miscible organic solvent to facilitate faster transport. Two phase systems with immiscible solvents are of particular interest (Schewe et al. 2009; Cornelissen et al. 2011). There are many parameters to consider when choosing a solvent: availability and price, the biocompatibility towards the enzymes and the host, low water solubility, substrate and product capacity, flammability, low emulsion forming tendency and in cofactor dependent reactions low NADPH consumption rate (Schewe et al. 2009). The solvent used also needs to be industrially applicable, environmentally friendly as well as non-hazardous. Generally, biocatalysis is better in organic solvents with an octanol/water partition coefficient (logP) greater than 4 (Laane et al. 1987). All the mentioned criteria lead to the options of using solvents such as hexadecane and bis(2-ethylhexyl)phthalate like in the case of styrene oxidation (Panke et al. 2000). Ethyl oleate was applied as a second phase in 1.5 L bioreactors to circumvent the low solubility of the substrate in the case of progesterone hydroxylation by CYP3A4 expressed in *Y. lipolytica*, increasing the final product concentration 5-fold to around 230 µM corresponding to 80 mg/L (Braun et al. 2012). On the other hand, the viscosity of phthalates can prevent the dispersion of the substrate and decrease the overall activity by diffusion limitations as well as the oxygen transfer rate at a given power input (Panke et al. 2002). The use of solvents also brings the concern about safety into the

picture, especially in combination with high oxygen supply and to address this explosive proof reactors have been designed at pilot scale (Schmid et al. 1998a).

2.4.3 Inhibition and toxicity

In reactions catalyzed by P450s not only the potential solvent but also the substrate and product are in many cases hydrophobic and toxic to the cells and inhibitory to the enzyme. The host selection should be done carefully, exemplified by *B. subtilis* 3C5N showing significantly higher tolerance towards the substrate 1-hexene compared to *E. coli* DH5α (Siriphongphaew et al. 2012). An efflux pump knock-out *E. coli*, enabling heme and substrate accumulation inside the cell improved the stability and reaction performance (up to 7 fold increase of product concentration) in the case of hydroxylation of compactin, vitamin D3 and 4-cholesten 3-one (Fujii et al. 2009). In a study of 10 recombinant P450s belonging to CYP1, CYP2 and CYP3 families, 13 substrates were tested of which 6 showed substrate inhibition in the range of 5-24 µM corresponding to 2-9 mg/L (Lin et al. 2001). Assuming a batch process with no engineering solutions applied, the tolerance would have to be improved by 3 orders of magnitude. Depending on the chemical properties of the molecules different approaches can be taken. Inhibition by the substrate can be overcome by *in situ* substrate supply (ISSS) using continuous supply of the substrate by a feeding strategy or by resins or solvents. Regarding the product *in situ* product removal (ISPR) can be applied by similar approaches (Lye and Woodley 1999; Woodley et al. 2008; Dafoe and Daugulis 2014). Product removal by resins was applied to steroid hydroxylation by CYP106A2 and enabled reuse of the catalyst (Zehentgruber et al. 2010a). Extractive fermentation with isopropyl myristate was applied in the case of artemisinic acid production by *Saccharomyces cerevisiae* reaching a product concentration of 25 g/L, an improvement by 10 g/L due to change of feed composition and introduction of the second phase (Paddon et al. 2013).

2.5 Improving space-time yield (g/L/h) by effective reactor operation

2.5.1 Transport limitations between phases in a two-phase system

Other parameters that have been addressed in the use of two-phase systems are the maximum transfer rates of the hydrophobic compounds between the organic and aqueous phase. The transfer

rates of n-heptane, n-octane and n-decane to the aqueous phase were determined to 79, 64 and 18 mmol/L/h respectively (Schmid et al. 1998b). Calculated in g/L/h these numbers are in the same order of magnitude (lowest value corresponding to 1.8 g/L/h) as the target space-time yield of 2 g/L/h. However, addition of the surfactant bis(2-ethylhexyl) sulfosuccinate sodium salt (AOT) to a two-phase hexane/water emulsion increased the initial rates of the hydroxylation of camphor by P450cam using yeast alcohol dehydrogenase for cofactor regeneration, indicating transport limitations between the phases in the hexane buffer system (Ryan and Clark 2008). Total turnover numbers achieved for the monooxygenase was 28900 and for YADH regenerating NADH 11800.

2.5.2 Oxygen requirements

Hydroxylations by P450s require equimolar amounts of oxygen. One oxygen atom is introduced to the substrate and one is reduced to water. For the reaction to take place it is of utmost importance that molecular oxygen is available and that it can be supplied at a sufficient rate. Even though potential oxygen limitation has not been studied to a large extent within the field of P450s, intact *E. coli* expressing recombinant P450 for the hydroxylation of progesterone has been suggested to be limited by molecular oxygen (Shet et al. 1997). Nevertheless, this study was conducted in milliliter scale without agitation, and the P450 catalyzed reaction was only responsible for 1.5 % of the total oxygen utilized by the cell. Already 1947, resting cells without nitrogen source were suggested to consume 45 % of what cells in the same state consumed in the presence of nitrogen source (Armstrong and Fisher 1947). This could be explained by the higher K_m of oxygenases (10-60 µM) compare to the electron-transfer chain (1 µM) meaning that at low oxygen concentrations, the oxygen will be used for respiration instead of the catalytic reaction (Duetz et al. 2001). Optimization of biomass and activity will be one important step in the process development and this has been done in the case of whole-cell Baeyer-Villiger oxidation (Baldwin and Woodley 2006). This is illustrated by optimizing the height of the grey area in Figure 2.3, assuming that the O_2 supply is sufficient. Besides the overall reaction rate, dissolved oxygen concentrations can also effect the reaction in terms of regioselectivity (Schneider et al. 1998).

As a rule of thumb, oxygen transfer rates in industrial size fermenters are 100 mmol/L/h and that would be able to transfer oxygen to 1670 U/L. Assuming a product with 154 g/mol in molecular weight, this activity corresponds to a space-time yield of 15.4 g/L/h if the supplied oxygen is solely used for the catalytic reaction (and with 100 % reaction yield). Keeping the target for space-time yield in mind, 2 g/L/h, efficient use of oxygen is required since 13 % of supplied oxygen has to be used for the reaction. This leaves the remaining 87 % for the metabolism or growth of the cell and it is clear that there is no room for poor coupling efficiency consuming oxygen without product formation. Depending on the specific requirement of oxygen of the resting cell the oxygen availability also set the requirements for the specific activity of the cell and thereby also on enzyme activity and expression levels. The oxygen requirements in combination with solubility issues leading to high oxygen pressures and solvents in combination consist of a safety problem as discussed above.

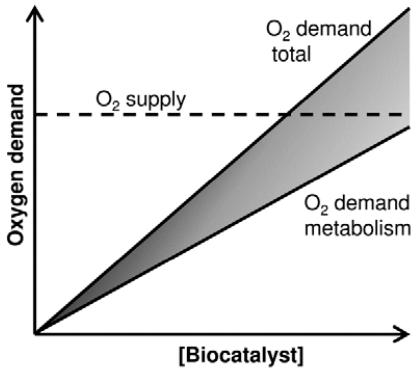


Figure 2.3 Oxygen demand vs biocatalyst concentration in whole-cell biocatalytic oxidation process. Dashed line represents possible O_2 supply from industrial sized fermenters, the lower solid line represents O_2 requirements for cell metabolism and the higher solid line the total O_2 demand for cell metabolism and P450 catalyzed reaction. (Reconstructed from Baldwin and Woodley (2006))

There are, however, alternatives to increase the oxygen transfer rate to the aqueous media if the specific activity per g cdw and catalyst concentration cannot be optimized enough in relation to oxygen supply. This can be done by increased oxygen pressure in the reactor by elevated pressure, supply of pure oxygen instead of air, modified oxygen supply configuration and complete reactor

configurations. E.g. the use of a bubble column, where air is released in fine bubbles through a sintered glass in the bottom of the reactor increased the activity of a whole-cell Baeyer-Villiger oxidation reaction (Hilker et al. 2004). Another example is bubble-free aeration using membranes of which the mass transfer characteristics have been described and equations presented (Cote et al. 1988). The technique was exemplified in a biocatalytic reaction enabling a reduction of the reactant feed rate with one third while still keeping the same reaction rate (Lynch et al. 1997). A circulation loop with a glass tube containing dead-end hollow gas-permeable fibers controlling the dissolved oxygen tension (DOT) with the oxygen pressure inside the fibers was used to prevent the volatile substrate fluorobenzene from evaporation.

2.6 Perspectives

There is no doubt that P450 monooxygenases perform excellent chemistry and based on that have huge potential within white biotechnology. Despite this fact and that lots of research have been done within the field not many industrial processes with P450s have been implemented. P450s are a good example of where chemists, biologists and chemical engineers need to work together in order to reach industrial implemented processes and that the idea of process development and identification of bottlenecks at an early stage can help to direct efforts. In order to improve a process that doesn't meet the requirements for being economically viable the bottlenecks first need to be identified. However, it is not easy to distinguish between parameters that at first glance depend on each other. The biological parameters important for whole cell P450 catalyzed reactions are summarized in Table 2.3 and numbers achieved for P450 systems are presented.

Table 2.3 Status of biocatalyst related parameters. Assumptions made throughout the text and also in the table is 1) Mw (P450 BM3) 118 kDa, 2) Mw (substrate) 138 g/mol, 3) Cell density 13 g cdw/L . Stability expressed here as time (h) or total number of product formed per enzyme or cofactor (TTN). However, following Michaelis Menten kinetics, reaction rate decreases over time but for simplicity calculated to be consistent over time.

PARAMETER			Current status	Reasonable improvement
Biocatalyst related parameters	Specific activity (U/g cdw)	Enzyme expression (nmol/L, nmol/g cdw)	12500 nmol/L (Pflug et al. 2007) 943nmol/g cdw	10x (Pflug et al. 2007)
		Enzyme activity (s^{-1})	>1 s^{-1}(Whitehouse et al. 2012)	20-100x (Malca 2012; Lewis and Arnold 2009)
		Cofactor regeneration (U/g cdw) including electron transport (s^{-1})	Calculated: 500-1000 U/g cdw (Duetz et al. 2001; Meyer et al. 2006; Blank et al. 2008) Oxygenases: 200 U/g cdw (Bühler and Schmid 2004) P450: 26 U/ g cdw (Fasan et al. 2011)	6-10x (Lee et al. 2013; Schewe et al. 2008)
		Coupling efficiency (%)	>98% (Fasan et al. 2007)	6x (Fasan et al. 2007)
	Stability	Cell (h)	(resting cell) >90h (Braun et al. 2012)	
		Monooxygenase (TTN, h) Reductase (TTN, h)	(CYP102A1 mutants w various substrates) 2200-30000 (Maurer et al. 2005)	10-20x (Maurer et al. 2005; Eiben et al. 2007; Salazar et al. 2003)
		Cofactor (TTN, h)	Ketoreductase (NADPH) 3400 supporting 61 U/g cdw (Schwaneberg et al. 2000) P450 (NADP+, *in vitro)* 280-1270 (Maurer et al. 2005)	

As can be seen in the table the prerequisites of successful whole-cell P450 biocatalytic (Maurer et al. 2005; Eiben et al. 2007; Salazar et al. 2003) processes are sufficient to reach target metrics (final product concentration and space time yield or biocatalyst yield) when parameters are analyzed individually. However, an efficient use of resources available is required.

In Figure 2.4 the importance of the weakest chain in the reaction system is illustrated and the influence it has on the economic potential illustrated by space-time yield and final product concentration. The biological parameters influencing specific activity are summarized in the lower left graph in the figure and following the figure clockwise the influence of these parameters on economic metrics are presented. The specific activity in combination with the cell concentration determines the space-time yield, which, along with the stability of the cell, influences the final product concentration. The final product concentration along with applied catalyst concentration subsequently determines

the biocatalyst yield for a resting cell process where the number of recycles also needs to be accounted for. What also can be seen is that stability of the system plays a crucial role, and once again it should be stressed that it is the stability under process relevant conditions that matters.

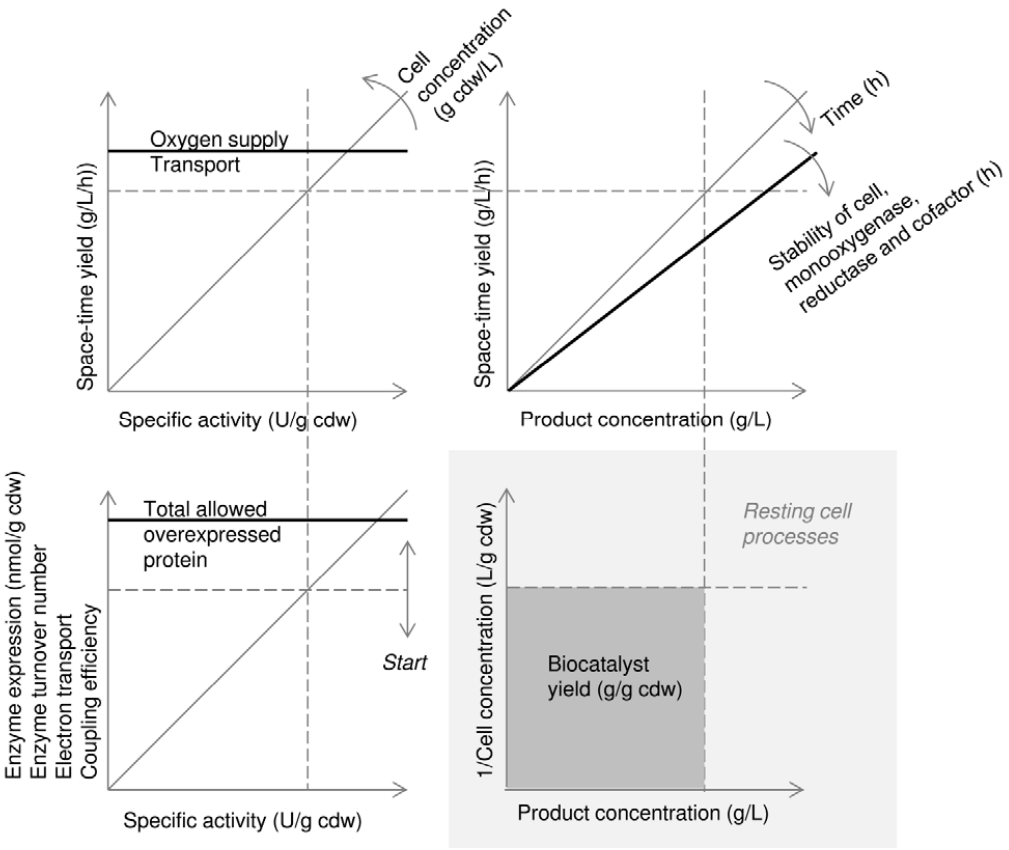


Figure 2.4 Influence of the typical biological parameters presented in P450 whole-cell processes on economic important parameters and limitations. Starting in the lower left corner the rate related parameters are listed (enzyme expression, enzyme turnover number, electron transport and coupling efficiency) influencing the specific activity that in the upper left corner together with the cell concentration results in the space-time yield. The space-time yield can be limited by the possible oxygen supply by the reactor and the transport of reactants between phases or across the cell membrane. From the space-time yield, the possible final product concentration is determined by the stability of the whole-cell system including the host cell, the monooxygenase, reductase and cofactors, shown in the upper right corner. The lower right corner illustrates how the cell concentration together with the final product concentration determines the biocatalyst yield for a

resting cell process where the number of recycles also needs to be accounted for. Thick lines indicate the limits to the metrics.

2.7 Guidelines

Biological limitations of P450 catalyzed whole cell reactions have previously been discussed in individual studies. In this chapter, quantitative comparisons of individual parameters in relation to economic metrics to guide the development of economically feasible processes have been made. Recommendations for successful development of P450 catalyzed processes for synthetic applications can be summarized as follows:

General:

- The main target for P450 catalyzed reactions are recommended to be high value molecules (fine chemicals and pharmaceuticals), complementary to existing chemical routes. For these high value products, k_{cat} values for many P450s are promising, assuming no other limitations. However, if the process is aiming for production of low range bulk chemicals the activity requirements of the enzyme increase by two orders of magnitude and only the P450s with the highest turnover numbers reported so far would be sufficient.

Biological:

- The stability of the catalyst is crucial, both in terms of cell, enzyme and cofactor. It can be concluded that turnover numbers in the higher range of what has been published would be enough if they could be achieved *in vivo* and stable over 24 h. A lower stability, meaning that product concentrations and biocatalyst yields need to be reached in a shorter time, would inevitably translate into higher demands on rates.
- Carefully selected host cell systems (e.g. natural P450 expressing hosts) for a robust host able to handle expression, reactive oxygen species and simplify transport across membrane are recommended.
- Cell and metabolic engineering to various extents, (tuning expression especially in multicomponent systems, balancing of redox cofactors and energy metabolites) to fully utilize

the potential of the catalyst. However, this approach is very resource demanding and the extent of the work needs to be carefully balanced to the gain expected. Approaches and tools for development of optimal industrial strains have recently been summarized by Van Dien (2013).

- The coupling efficiency needs to be maximized to utilize available cofactor efficiently, minimize toxic byproducts and economical use of oxygen supplied.

Process:

- Product localization determines product recovery and if cell disruption is necessary a growing cell process is favored due to the higher economic demands on the resting cell process. Growing or resting cells needs to be determined on a case-by-case basis and factors like stability and product profile can also influence the decision.
- Using process knowledge and constraints gained from substrate and product properties regarding solubility, toxicity and transport should be taken into account when designing the overall process from the beginning, instead of designing a process purely based on the P450 in mind as illustrated in Figure 2.5.

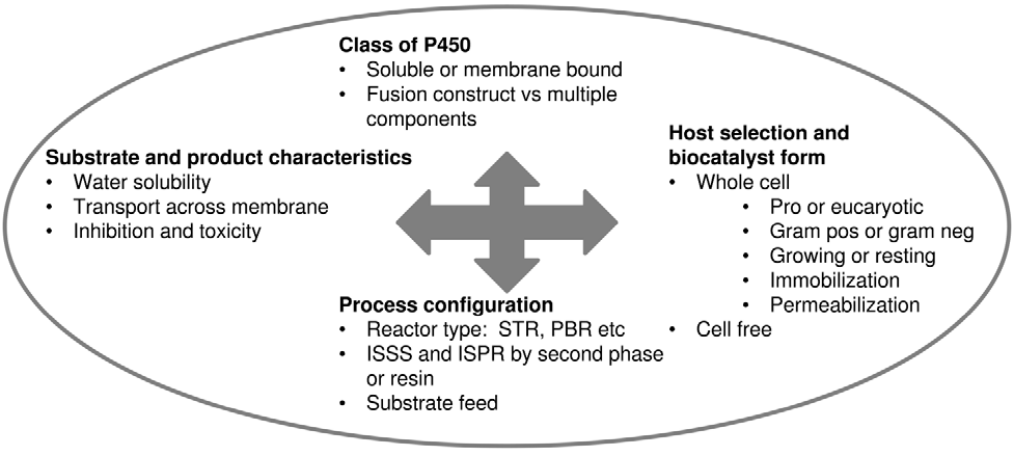


Figure 2.5 The development of biocatalytic P450 processes needs to consider the overall picture to guide efforts and thereby enable more focused process development.

3 Case study I: CYP153A expressed in *E. coli* catalyzing the ω-hydroxylation of dodecanoic acid

The model system chosen for the first case study represents an artificial P450 fusion construct expressed in a naturally non-P450 expressing host represented by *E. coli.* A fusion construct consisting of the P450 heme domain and the electron transporting redox partner in a single protein is favorable for high expression levels and has, in many cases, been suggested as a promising approach for efficient electron transfer. *E. coli* is the most applied organism in research laboratories with well explored genetics and is easy to handle. This model system was chosen based on a product with commercial interest and a soluble protein with reasonably high activity, selectivity and coupling efficiency. The fusion construct was created and characterized in Professor Hauer's group, University of Stuttgart, Germany, who also kindly supplied the plasmid. The CYP153A heme domain from *Marinobacter aquaeolei* fused to the reductase domain of CYP102A1 from *Bacillus megaterium* expressed in *E. coli* was applied to identify the bottlenecks of a resting whole cell catalyzed process. This was done by characterization of the biocatalyst and reaction system. The P450 chimera has been shown to regioselectively hydroxylate fatty acids of medium chain length. Terminal hydroxylated fatty acids can be used in the field of high end polymers, in fine chemicals and in the cosmetic and fragrance industry. The industrial relevant *E.* coli strain HMS174 was chosen as suitable host and the fermentation process was developed in Gerald Striedners lab, BOKU, Vienna, Austria. The work presented in this chapter is based on Paper II.

3.1 Introduction

The complex nature of P450s, including cofactor dependence and the requirement of redox partners, is associated with several challenges for their application in industrial processes (O'Reilly et al. 2011). Whole cells are the preferred biocatalyst formulation since they provide the ability to regenerate cofactors, mainly via the pentose phosphate pathway, the tricarboxylic acid cycle and the transhydrogenases system (Lee et al. 2013), as well as increased stability of the enzyme due to a protected environment. In the previous chapter, challenges for P450 catalyzed whole cell processes to

reach economic feasibility and industrial implementation have been discussed. Based on the potential limitations reported for whole cell processes, here a strategic approach to identify the bottlenecks in a P450 catalyzed reaction using resting cells have been applied. The suggested limitations were described to be substrate and product related inhibition and toxicity, stability (of P450, host cell and cofactor), cofactor regeneration and transport limitation between phases and across the cell membrane (Lundemo and Woodley 2015). The aim of this chapter is to validate the literature based limitations using a well-developed model system, terminal hydroxylation of medium and long chain fatty acids by a P450 fusion construct.

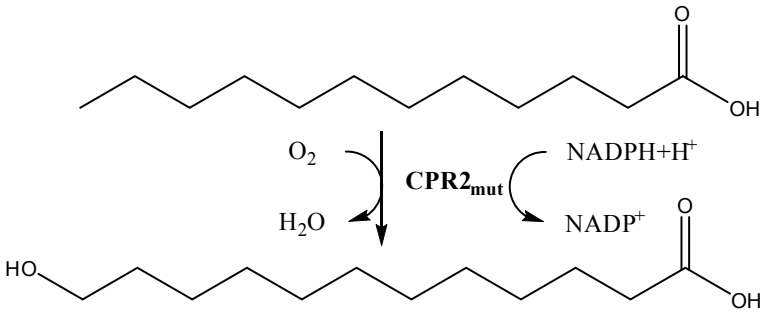
Terminal hydroxylated fatty acids (ω-OHFAs) and α,ω-dicarboxylic acids (α,ω-DCAs) derived from medium and long chain length fatty acids are key intermediates used as building blocks for the synthesis of valuable bioplastics, and precursors of high end polymers in the chemical industry (Liu et al. 2011). Oxygenated fatty acids can additionally be used in the cosmetics area for production of perfumes and for pharmaceutical applications such as anticancer agents. ω-OHFAs and α,ω-DCAs can be produced both by chemical and biological routes. Selective oxidations of alkanes by the chemical routes suffer from poor selectivity (Labinger 2004). A promising biological alternative is the biological route catalyzed by P450s, originating both from eukaryotic yeast strains and bacterial strains as exemplified below. Significant strain engineering was applied to *Candida tropicalis* expressing CYP52A. The improved host reached a final product concentration of 174 g/L of 14-hydroxytetradecanoic acid and 6 g/L of 1,14-tetradecacanedioic acid after 148 h of biotransformation mainly by avoiding overoxidation by eliminating the β-oxidation pathway (Lu et al. 2010).

Alternatively to the yeast CYP52, one family of enzymes shown to hydroxylate short and medium chain fatty acids but also alkanes, primary alcohols, and limonene, is the bacterial CYP153 (Malca 2012; Scheps et al. 2011; Van Beilen et al. 2005; Bordeaux et al. 2011). The three component CYP153A6 from *Mycobacterium sp.* strain HXN-1500, was expressed in *E. coli* resulting in 6 g/L (S)-perillyl alcohol from (S)-limonene in a two-liquid phase system, coexpressing AlkL for improved substrate transport across the membrane (Cornelissen et al. 2013). The side product formation was reduced to 8 % by changing host from *P. putida* to *E. coli*. In another study, the same three

component CYP153 system was expressed in *E. coli* and applied for production of octanol in a resting cell transformation at 1 mL scale leading to a final product concentration of 8.7 g/L of after 24 h (Gudiminchi et al. 2012). Furthermore, a fusion construct with CYP153A13a fused to reductase domain of P450RhF expressed in *E. coli* was previously demonstrated to hydroxylate n-octane to 1-octanol at a 5 mL scale, however, not exceeding the product concentration above (Bordeaux et al. 2011).

Within this subfamily, CYP153A from *Marinobacter aquaeolei* was shown to catalyze the terminal hydroxylation of medium and long chain-length fatty acids with high selectivity (Malca 2012). A chimera protein was constructed consisting of the heme domain of CYP153A from *M.aquaeolei* fused to the reductase domain of CYP102A1 from *Bacillus megaterium*. This self-sufficient chimera $CYP153A_{M.aq.}$-CPR_{BM3} mutant G307A (termed $CPR2_{mut}$) with an increased activity and coupling efficiency compared to the wild-type, is a soluble protein with more than 95 % ω-regioselectivity towards saturated fatty acids in the range of $C_{12:0}$-$C_{14:0}$ (Malca 2012). $CPR2_{mut}$ was recently applied using a whole cell *E. coli* host in a successful biotransformation producing 12-hydroxydodecanoic acid methyl ester (Scheps et al. 2013). The methyl ester instead of the fatty acid was used as substrate and applied in excess as a second liquid phase reaching a final product concentration of 4 g/L.

To further improve the economic potential of terminal hydroxylation of medium and long chain length fatty acids by this whole cell *E. coli* catalyst a better understanding of the limitations is required. This study focuses on the characterization of the artificial fusion construct $CPR2_{mut}$ expressed in *E. coli* for the production of 12-hydroxydodecanoic acid (Scheme 3.1). This is done with the intention to identify and overcome bottlenecks of the reaction system preventing this whole cell P450 catalyzed process from reaching economic targets by studying whole cell biotransformations, in parallel with *in vitro* studies.



Scheme 3.1 ω-hydroxylation of dodecanoic acid to 12-hydroxydodecanoic acid catalyzed by the fusion construct $CPR2_{mut}$ (CYP153A heme domain fused to the reductase domain of CYP102A1).

3.2 Materials and methods

The chosen model system used in this study was the terminal hydroxylation of dodecanoic acid performed by CYP153A G307A from *Marinobacter aquaeolei* fused to the reductase domain of CYP102A1 from *Bacillus megaterium* (termed $CPR2_{mut}$) (Scheme 3.1). The fusion protein was expressed in the industrially feasible *E. coli* strain HMS174 (DE3) for whole cell biotransformations, in *E. coli* BL21(DE3) for enzyme purification and in a knock-out BL21 strain for the beta oxidation pathway (*ΔfadD*) for product inhibition studies.

3.2.1 Chemicals, bacterial strains and plasmids

Yeast extract and tryptone were purchased from Nordic Biolabs (Täby, Sweden), dodecanoic acid from Merck KGaA (Darmstadt, Germany) and all other chemicals were from Sigma-Aldrich (Schnelldorf, Germany). Plasmid pET-28a(+) (Novagen, Madison, WI, USA) and L-rhamnose inducible plasmid pJOE4782.1 (kindly provided by Dr. Josef Altenbuchner (Stumpp et al. 2000)) with the fusion construct $CPR2_{mut}$ inserted were generated at the ITB, University of Stuttgart, Germany. *E. coli* strain HMS174 (DE3) was obtained from the Striedner lab (BOKU, Vienna, Austria). *E. coli* strain BL21 (DE3) was purchased from Novagen (Madison, WI, USA) and the BL21 (*Δ fadD*) (strain with knocked-out beta-oxidation pathway) was kindly provided by the ITB, University of Stuttgart, Germany.

3.2.2 Fermentation

A seed culture with LB media supplemented with kanamycin (30 µg/mL) was inoculated from a -80 °C glycerol stock and grown at 37 °C, 180 rpm until late exponential phase in shake flasks. The seed culture was used to inoculate a fermenter with 1 L working volume (Infors AG, Bottmingen, Switzerland) and fermentation was performed by glucose limited fed-batch with a growth rate of 0.1 h^{-1} (Marisch et al. 2013). Fermentation was controlled by Iris software (Infors AG, Bottmingen, Switzerland) and feed was initiated when the carbon source from the batch media was depleted, indicated by a pO_2 spike. Protein expression was induced by 0.5 µmol IPTG per g cdw one generation after feed start and the process continued for 3 additional generations. Temperature was decreased at the time of induction from 37 °C to 30 °C, pH was set to 7.2 and controlled by addition of 14 % NH_4OH and pO_2 set to 30 % was controlled by agitation. Foam was controlled by addition of 0.5 mL antifoam 204 (Sigma-Aldrich) per liter media. Cells were harvested by centrifugation (4000xg, 25 min), washed and resuspended in 100 mM potassium phosphate buffer pH 7.4, supplemented with 20 mM glucose and 1 % glycerol and stored gently shaking in 4 °C until further use.

An initial seed culture was also used to inoculate 2 L Erlenmeyer shake flasks using TB medium for cell cultivation and protein expression aerobically in shaking incubators (Multitron, Infors HT, Bottmingen, Switzerland). Cells were grown at 37 °C, 180 rpm until OD_{600} of 0.7-1 was reached before induction and protein expression at 25 °C, 180 rpm. After 16-20 h of protein expression, the cells were harvested and treated as described above.

3.2.3 Protein purification

The cells were disrupted using an EmulsiFlex-C5 (Avestin, Mannheim, Germany) or by sonication on ice (3x2min, 1 minute interval (Heinemann, Schwaebisch Gmuend, Germany)). The crude cell extracts suspended in 50 mM Tris-HCl buffer pH 7.4 were centrifugated (17 000 rpm, 45 min, 4 °C) and the supernatant containing the soluble P450 protein was recovered. The protein was purified using an Äkta system (GE Healthcare Biosciences, Uppsala, Sweden), with a weak anion exchange column (30 mL) packed with Toyopearl DEAE 650 M (TOSOH, Stuttgart, Germany) at a maximum flow rate of 10 mL/min. The binding buffer consisted of 50 mM Tris-HCl buffer (pH 7.4) and for elution a step gradient

of 50 mM Tris-HCl containing 1 M NaCl was used. The protein elution was detected by a characteristic absorbance at 418 nm, in addition to the total protein detection at 280 nm (Sligar et al. 1979) and eluted at a concentration of 250 mM NaCl. After purification, the protein was concentrated using 100 kDa cut-off Vivaspin tubes (Sartorius, Goettingen, Germany) and aliquots were stored at -20 °C until further use.

3.2.4 *In vitro* substrate and product inhibition studies

Substrate and product inhibition were investigated using purified enzyme (0.5 µM and 1 µM) in a final volume of 300 µL in eppendorf tubes with 50 mM potassium phosphate buffer, pH 7.4, 1 mM NADPH cofactor and a cofactor regeneration system (5 mM glucose-6-phosphate, 1 mM $MgCl_2$, 12 U/ml glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides*). For substrate inhibition studies the dodecanoic acid concentration was varied between 0.1 and 2.5 mM. The product inhibition experiments were performed with 1 mM dodecanoic acid and 0-2 mM 12-hydroxydodecanoic acid. The substrate and product were dissolved in DMSO prior to addition and the final DMSO concentration was kept at 5 % and not exceeding the water solubility limit. The reactions were performed at 30 °C, 700 rpm stirring, starting with the addition of 1 mM NADPH.

3.2.5 Resting cell biocatalytic reactions in Erlenmeyer flask

Studies of parameters believed not be influenced by scale were performed in 100 mL Erlenmeyer flasks with a catalyst concentration of 50 g cww/L. Carbon source (20 mM glucose and 1 % glycerol) was added at time point 0, 4 h and 8 h of biotransformation. Biocatalytic reactions were performed at 30 °C and 180 rpm.

3.2.5.1 In vivo substrate and product inhibition studies

In vivo substrate and product inhibition studies were performed similarly to the *in vitro* studies. Biotransformations with 50 g cww/L resting whole cells in shake flasks were used and various initial substrate concentrations for substrate inhibition studies and a constant substrate concentration with various amounts of product added at time zero were applied for product inhibition studies. The substrate and product were pre-dissolved in DMSO and 5 % of DMSO was used as final concentration. Inhibition profiles were estimated from initial reaction rates.

3.2.5.2 Cofactor limitation

Cofactor limitation was explored by addition of external NADPH in stoichiometric concentrations (1.5 mM) or 3 mM from a pre-dissolved stock in 100 mM potassium phosphate buffer (pH 7.4). The cofactor was confirmed to be taken up by the cell by monitoring the absorbance by NADPH at 340 nm.

3.2.5.3 Transport limitation

Transport limitation was examined by different permeabilization methods prior to the biocatalytic reaction. Both mechanical and chemical methods were applied. Frozen cells were spun down and pellet frozen in -20 °C over night. Acetone treated cells were incubated with 5 % acetone during 2 minutes while vortexing. Sonication was performed during 1x2 minutes (amplitude 60 %, 0.5 s cycles) (UP400 S, Hielscher Ultrasonic GmbH, Teltow, Germany). Following permeabilization treatment (acetone treatment and sonication) cells were spun down and the pellet was resuspended in 100 mM potassium phosphate buffer pH 7.4.

3.2.6 Resting cell biocatalytic reactions in bioreactor

To expose the biocatalyst to more industrial relevant conditions, in terms of stirring and aeration, biocatalytic reactions were also performed in a stirred tank reactor (Infors Multifors 2 (Infors AG, Bottmingen, Switzerland)). Settings controlled via Iris software were pO_2, temperature and pH with setpoints 30 % (controlled by stirrer), 30 °C and 7.4 (controlled by 10 % H_3PO_4 and 5 M NaOH) respectively. Aeration was set to 3.75 vvm. Antifoam 204 was added according to need.

3.2.7 Analytical methods

3.2.7.1 P450 determination

The concentration of correctly folded P450 was measured by CO differential spectral assay (Omura and Sato 1964). Samples from fermentations and biotransformations were stored in the form of frozen cell pellet until analyzed. Cell pellet was resuspended in 2 mL 100 mM potassium phosphate buffer and a spatula tip of sodium hydrosulfite was added to the resuspension and incubated on ice for 10 min. The resuspension was split into two cuvettes, of which one was treated with carbon monoxide for 1 min before the differential spectra was measured between 400 and 500 nm (UV-1800, Shimadzu Corporation, Kyoto, Japan).

3.2.7.2 Cell dry weight determination

Pellets from 1 mL fermentation or biotransformation samples were frozen and, after thawing, resuspended in an equal volume of 100 mM potassium phosphate buffer, pH 7.4 and filtered through preweighed 0.22 µm PES membrane filter (Frisenette, Knebel, Denmark) by application of vacuum. The filters were dried in a microwave and left to equilibrate in a desiccator before weighing both prior to, and after, application of the sample.

3.2.7.3 GC analysis

Reaction progress was monitored by GC-FID (Clarus 500 Gas Chromatograph, PerkinElmer, Waltham, MA, USA) using a Elite-5 column (PerkinElmer, Waltham, MA, USA) and helium as carrier gas. Alternatively, the mass spectra were collected on a GC-MS QP-2010 instrument (Shimadzu, Japan) equipped with a DB-5 MS column (30 m×0.25 mm×0.25 µm) (Agilent Technologies, Santa Clara, CA, USA) with helium as carrier gas. Mass spectra were collected using ESI (70 eV). Samples were prepared by extraction in twice the sample volume with diethylether or tert-methyl butyl ether, the organic phase pooled and centrifuged and then left to evaporate followed by resuspension in equal volumes (60 µl) tert-methyl butyl ether and N,O-bis(trimethylsilyl)trifluoroacetamide containing 1 % trimethylchlorsilane before derivatization (20 min, 70 °C). Tridecanoic acid or decanoic acid were used as an internal standard. A linear temperature gradient from 180 °C to 300 °C (15 min) was applied after 1 minute at initial conditions. The injection volume was 1 µL and a split ratio of 20 was used.

3.3 Results

3.3.1 Catalyst characterization

To identify the limitations of the whole cell catalyzed ω-hydroxylation of dodecanoic acid by $CPR2_{mut}$ expressed in *E. coli*, the system was initially characterized at small scale (shake flasks). This part of the work focused on the limitations identified in general for whole cell P450 catalyzed reactions, related to the catalyst and reaction. The potential substrate and product inhibition was examined *in vitro* and *in vivo* to identify suitable reaction conditions. Furthermore, cofactor regeneration of the resting host cell and potential substrate transport limitations across the cell membrane were also investigated.

3.3.1.1 Substrate inhibition studies in vitro and in vivo

To determine potential substrate inhibition of the enzyme, *in vitro* reactions were performed using purified $CPR2_{mut}$ (1 µM) and different concentrations of dodecanoic acid (0.1-2.5 mM), starting the reaction with the addition of the cofactor NADPH. Initial rates were estimated after 2 minutes of reaction (Figure 3.1). At higher substrate concentrations (above 1 mM), the initial rate decreases indicating an inhibitory effect of the substrate. In Figure 3.1, a reciprocal plot used for detection of substrate inhibition is displayed (Cornish-Bowden 2012) and potential kinetic parameters resulting in the dashed line in Figure 3.1 are K_m 0.4, v_{max} 0.25 and K_{si} 2.

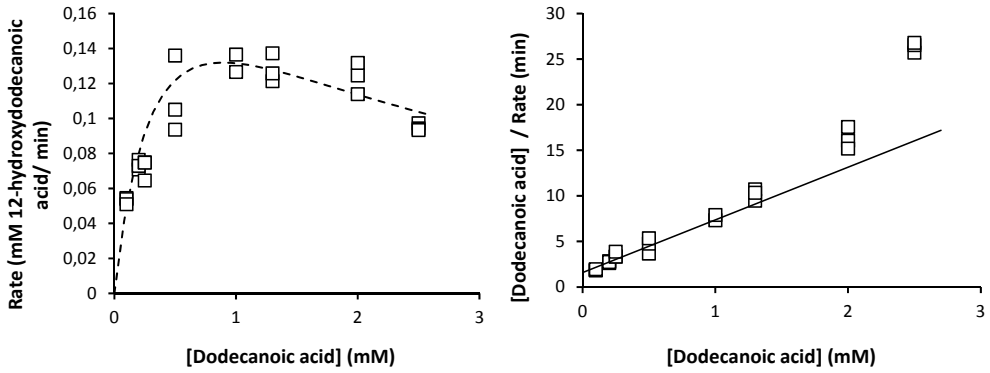


Figure 3.1 Left: Substrate dependence of initial product formation rate catalyzed by 1 µM of purified $CPR2_{mut}$. Trendline is indicated with a dashed line. **Right:** Reciprocal plot for detection of substrate inhibition (Cornish-Bowden 2012). At high substrate concentrations deviations from the linear dependence (solid line) predicted from concentrations lower than or equal to 1 mM can be seen.

To investigate whether the same substrate inhibition trend could be observed using the whole cell catalyst, the effect of increasing substrate concentrations was also investigated *in vivo,* using resting cells. Figure 3.2 illustrates the initial reaction rate at various substrate concentrations in the range of (0.5–5 mM) using 50 g cww/L. The initial rate was estimated after 1 h of biotransformation in shake flasks. *In vivo* results confirm the indicated substrate inhibition *in vitro*.

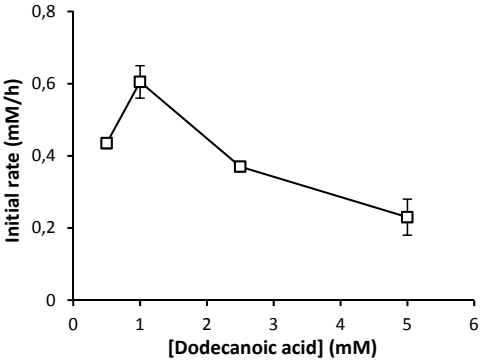


Figure 3.2: Whole cell biotransformation with increasing concentrations of dodecanoic acid (using 5 % DMSO). Experiments performed in shake flasks using 50 g cww/L in duplicates and error bars are 1 σ.

Substrate inhibition was further confirmed in bioreactors, where transformations with resting cells were performed with 5, 10 and 15 mM substrate concentration. The lowest concentration (5 mM) displayed 95 % conversion within 1 h of reaction whereas the transformation performed with the highest substrate concentration (15 mM) did not show any conversion during the 22 h reaction course (Figure 3.3). The 5 and 10 mM experiments did not show any significant difference in initial rate. However, when 10 mM of substrate was applied, the reaction rate slowed down after 50 % conversion corresponding to 5 mM product formed and did not reach complete conversion which potentially could indicate a negative effect of the product formed. The initial rate of experiments performed in shake flasks and in stirred tank reactors (Figure 3.2 and Figure 3.3) differs in favor of reactor experiment and this has also been observed previously, and is believed to be due to enhanced mixing in the stirred tank reactor (Kiss et al. 2015). The effect in this case could alternatively be attributed to the cells originating from different fermentation batches.

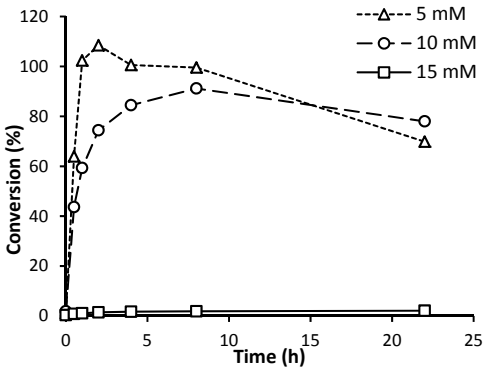


Figure 3.3 Conversions for transformation in bioreactor with increasing substrate concentrations (5, 10 and 15 mM). Experimental conditions: 50 g cww/L, 100 mM potassium phosphate buffer pH 7.4. The substrate was dissolved in DMSO and applied using a final DMSO concentration of 5 % (v/v).

3.3.1.2 Product inhibition studies in vitro and in vivo

To evaluate if the reason for the slower conversion rate after reaching high product concentration could be product inhibition, *in vitro* reactions were performed using a constant concentration of substrate (1 mM) and different initial concentrations of the product 12-hydroxydodecanoic acid (0-2 mM) (Figure 3.4). The decreasing initial rate at increasing product concentrations indicates product inhibition using 1 µM and 0.5 µM of purified enzyme. No reaction took place at product concentrations above 1 mM and 1.5 mM for enzyme concentrations of 0.5 µM and 1 µM, respectively. These results indicate that the product inhibition is dependent on the biocatalyst concentration and not only the absolute product concentration, implying that with a lower enzyme concentration the negative effect appears at lower actual concentrations of product. Without a detailed mechanistic investigation, this could potentially be explained by an irreversible binding of the product to the active site of the enzyme, thereby making the inhibition or inactivation dependent on the ratio of product and enzyme.

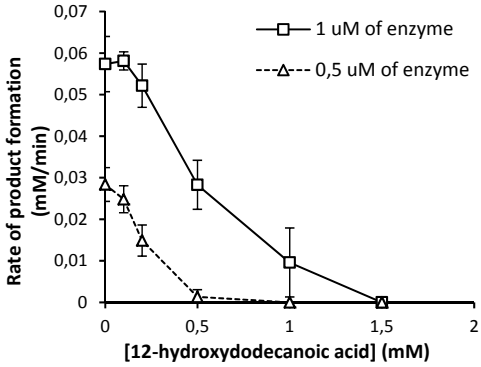


Figure 3.4 Influence of increasing initial product concentration (0-2 mM) on initial rates. The reaction was performed *in vitro* and initiated by the addition of 1 mM of dodecanoic acid. The substrate was dissolved in DMSO and applied using a final DMSO concentration of 5 % (v/v).

Potential product inhibition was also evaluated *in vivo* using resting whole cells in shake flasks performing biotransformations of 1.5 mM of initial dodecanoic acid as substrate (Figure 3.5). The *E. coli* strain BL21 (*ΔfadD*) was used in order to avoid any consumption of formed product by the β-oxidation pathway. What could be observed was that the rate of product formation after 2 h of reaction decreases with increasing concentration of product at time point 0. In agreement with the *in vitro* results, the presence of the product, 12-hydroxydodecanoic acid, is inhibitory also to the whole cell catalyst.

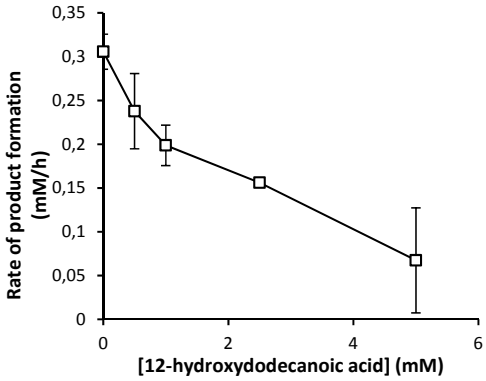


Figure 3.5 Product inhibition profile *in vivo* after 2 h biotransformation using 1.5 mM dodecanoic acid and increasing initial product concentration performed in shake flasks. The substrate was dissolved in DMSO and applied using a final DMSO concentration of 5 % (v/v).

3.3.1.3 Cofactor regeneration

One of the reasons for using a whole cell system for cofactor requiring catalysis is the ability of metabolically active cells to regenerate nicotinamide cofactors. This has however been suggested to be a potential limitation in an overexpressed host (Lundemo and Woodley 2015; Bernhardt and Urlacher 2014; Siriphongphaew et al. 2012). We investigated the potential limitation of resting *E. coli* to regenerate sufficient NADPH for the desired biocatalytic reaction. Resting whole cell biotransformation was performed in shake flasks, at a substrate concentration of 1.5 mM (Figure 3.6). Different amounts of NADPH were added to the biotransformations and the initial rate was evaluated. The highest initial rate was obtained when twice the stoichiometric amounts of substrate of NADPH was provided (3 mM) indicating that the cofactor regeneration of the resting cell is not sufficient to support the catalytic reaction, at achieved rates and with current coupling efficiency.

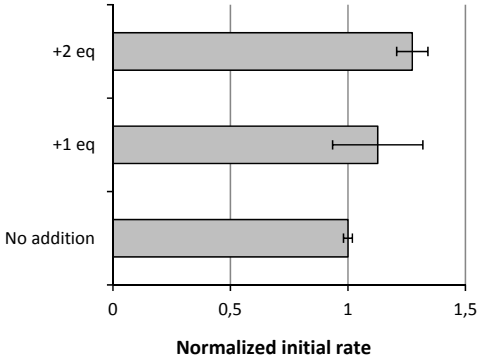


Figure 3.6 Influence of cofactor addition on biotransformations with resting cells in shake flasks. Reaction conditions: 1.5 mM dodecanoic acid and NADPH cofactor added at the start of the reaction.

3.3.1.4 Substrate transport limitation studies

The last potential limitation related to the biocatalyst investigated in this study was substrate transport across the cell membrane. Different permeabilization techniques (sonication, acetone treatment and freezing/thawing) were applied prior to resting cell biotransformations. Progress curves of the biotransformations using 1.5 mM dodecanoic acid are shown in Figure 3.7 with and without stoichiometric amounts of NADPH added. The highest final concentration was reached by intact cells with and without additional cofactor added. No permeabilization method with or without the addition of cofactor increased the reaction performance, indication that the reaction is not limited by substrate transport across the cell membrane.

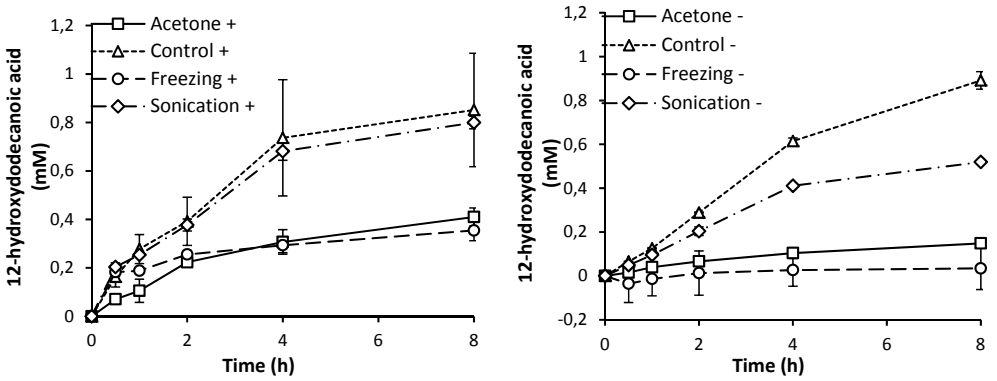


Figure 3.7 Progress curves of biotransformations of 1.5 mM dodecanoic acid in shake flasks after different permeabilization methods of the cell membrane. The experiments were performed in the presence (**left figure**) as well as in the absence (**right figure**) of stoichiometric amount of NADPH cofactor (1.5 mM) supplemented at the beginning of the reaction.

3.3.2 Process development

To study the reaction in a more easily controlled environment and with relevant agitation and oxygen supply further transformations processes were performed in a bioreactor, which is also the final intended reactor configuration for this type of biotransformation process.

3.3.2.1 Operating in a bioreactor

Progress curves of a biotransformation performed in a 1 L bioreactor using 5 mM substrate concentration is shown in Figure 3.8. Substrate inhibition was shown to occur *in vivo* in shake flask transformations already above 1 mM substrate concentration. However, the inhibition profile and reaction rate was shown to be different in stirred tank reactors compared to shake flasks as already discussed. What also could be detected at higher substrate concentration was the overoxidation to dicarboxylic acid. During the course of the reaction, the influence on cell viability was followed by measuring the cell dry weight and the enzyme inactivation was monitored spectrophotometrically by the absorbance at 450 nm of the reduced CO bound form of the correctly folded heme domain (Omura and Sato 1964) (Figure 3.8). It can be seen that the whole cell is intact during the reaction

course but 50 % of the correctly folded enzyme has been lost already after 2 h. This implies that increased stability of the P450 fusion construct is essential for further development of the process.

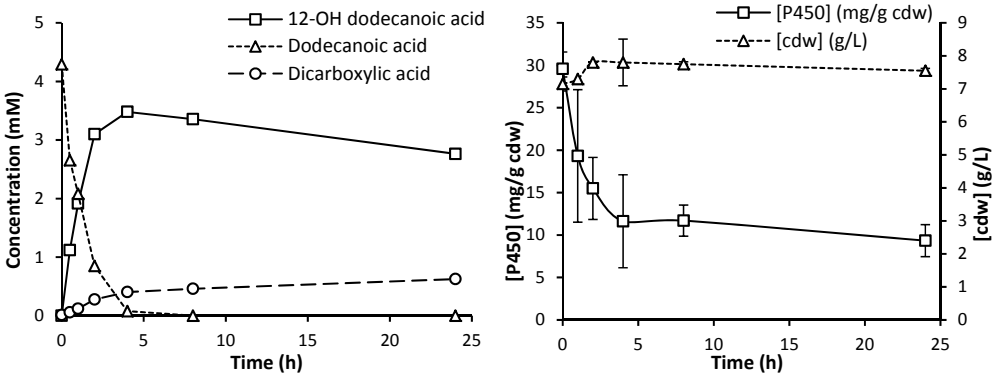


Figure 3.8 Left: Progress curve of biotransformation performed in 1 L bioreactor. Reaction conditions: 50 g cww/L, 100 mM potassium phosphate buffer pH 7.4, 30 °C, pO_2 30 %, initial substrate concentration 5 mM. **Right:** Cell dry weight and correctly folded P450 during transformation performed in 1 L reactor.

3.3.2.2 Media supplements

To understand the preferred reaction conditions and mode of operation further experiments with and without carbon source and nitrogen source were performed. Supplemental carbon was found to be critical for the performance of the resting cells (Figure 3.9), most likely due to the requirement of carbon source for cofactor regeneration. Addition of nitrogen source (ammonium sulfate) to the resting cell reaction performed in 100 mM phosphate buffer (pH 7.4) did not show any effect on the catalytic performance, however, the cells started to increase in density measured by following the cell dry weight (Figure 3.9). Based on these results, the biocatalytic reaction could also be performed in a growing cell format. However, this was not investigated further in this study.

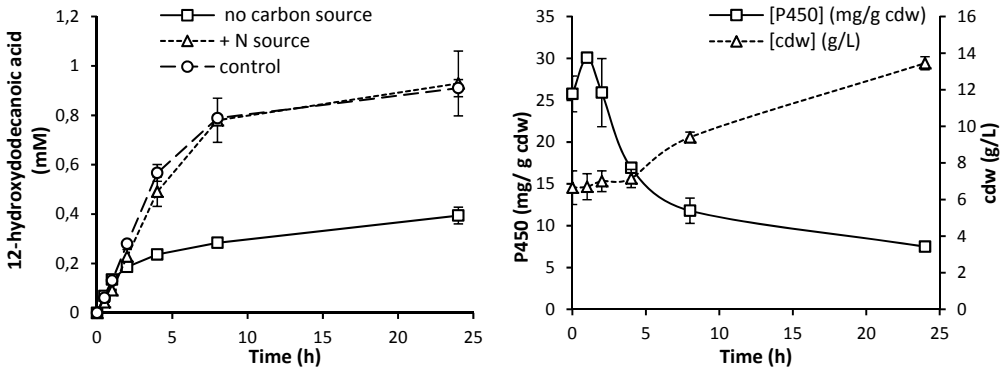


Figure 3.9 Left: Comparison of resting cell biotransformations with and without carbon and nitrogen source performed in shake flasks. Carbon source can be seen to be required to regenerate cofactors required for the biocatalytic reaction. **Right:** Addition of nitrogen source does not increase the reaction performance and enables cell growth seen by increasing biomass concentration. Data from a 5 mM transformation performed in a bioreactor. A positive effect on the stability of the P450 is indicated compared to the resting cell reaction without nitrogen source seen in Figure 3.8. (N – nitrogen)

3.3.2.3 Substrate supply

Substrate and product inhibition together with poor P450 stability and insufficient cofactor regeneration have been demonstrated as limitations for this reaction system. Different substrate feeding strategies were used as a simple and easily implemented method to avoid the substrate inhibition and potentially increase the stability, by e.g. eliminating the use of co-solvent. The commonly used batch process with substrate applied dissolved in DMSO (to a final concentration of 5 %) was compared with application of the substrate dodecanoic acid in solid form. In addition, a batch with solid application of substrate using cells pre-treated with 5 % DMSO and a fed-batch process (loading substrate dissolved in DMSO) were evaluated. The best conversion of substrate and the best rate of reaction were achieved by application of substrate in solid form (Figure 3.10). The reduced performance of the cells pretreated with DMSO indicates that the negative effect of the co-solvent can be explained by damage to the catalyst. However, the difference of the solid application of substrate to the cells pretreated with DMSO and the traditional batch process can most likely be

explained by inhibition by the substrate and product present in the aqueous phase when the co-solvent is present.

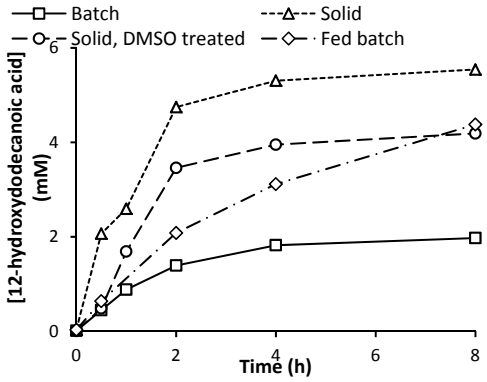


Figure 3.10 Biotransformation reaction performed in 1L bioreactor. Reaction conditions: 50 g cww/L, 100 mM potassium phosphate buffer pH 7.4, 30 °C, pO_2 30 %, initial substrate concentration in the batch 10 mM.

To study the solubility of dodecanoic acid and to understand the positive effect of the solid application of the substrate, an experiment was set up identical to the biotransformation in the bioreactor, with the exception that no cells were added. Samples were taken during the same time interval as during the biotransformation. The aqueous solubility under these conditions was shown to be approximately 1.5 mM (Figure 3.11). This is significantly lower than the final product concentration reached when solid feed was applied. The reported water solubility at 25 °C of dodecanoic acid and 12-OH dodecanoic acid is 12.76 mg/L (ChemSpider b) and 278 mg/L (ChemSpider c) respectively corresponding to 64 µM and 1.3 mM. This indicates that with our experimental setup higher solubility was reached. The achieved product concentration implies that the solubilization of the substrate continues during the reaction course and that the rate of dissolution is not limiting the rate of reaction.

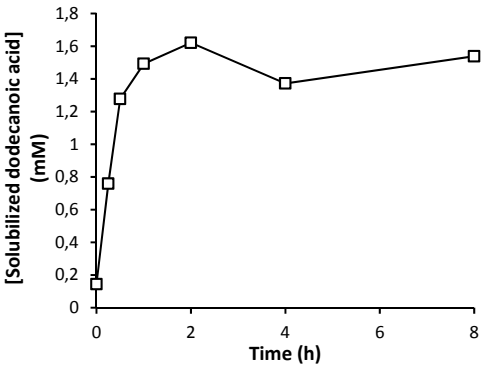


Figure 3.11 Solubility of 10 mM dodecanoic acid in 100 mM potassium phosphate buffer over time.

3.4 Discussion

In order to reach the requirements of an economically feasible biocatalytic process the concentration of product (and therefore substrate) need to be increased from what is normally used in research laboratories. In this chapter a methodological approach has been used to identify bottlenecks preventing us from reaching the concentration targets for an economically feasible process. Theoretically identified limitations defined in Chapter 2 have been the basis for the investigation. The P450 chimera consisting of the heme domain of CYP153A from *Marinobacter aquaeolei* fused to the reductase domain of CYP102A1 has been well studied. The heme domain was modified by protein engineering for increased activity and the regioselectivity of the enzyme was improved towards terminal hydroxylation (Malca 2012). By creation of a fusion construct the best prerequisites for high expression levels, efficient electron transport with a coupling efficiency of 73 % and high activity have been fulfilled (Scheps et al. 2013).

E. coli HMS174, an industrial relevant *E. coli* strain, known to have an enhanced plasmid stability compared to BL21 was used as model host in this study. A successful fermentation process for expression of the fusion construct has been established yielding 9 000 nmol P450/L for a fermentation process with a final cell density of 25 g cdw/L based on Marisch et al. (2013).

The focus of the biocatalytic reaction has been resting cell biotransformations, to be able to separate the reaction from growth. This mode of operation allows optimization of the biocatalytic reaction, e.g. by change of biomass concentration and also recycle of the biocatalyst. Limiting parameters are defined as substrate and product inhibition as well as enzyme stability and cofactor regeneration. The substrate inhibition has been addressed here with improved reaction performance using solid application of substrate, also benefitting from the avoidance of organic co-solvent. Furthermore, external supplement of cofactor was shown to be beneficial for the biocatalytic reaction but is obviously no economical viable option to improve the cofactor availability. The cofactor regeneration can be improved by coexpression of e.g. dehydrogenase. However, the consequence of lower possible expression levels of the P450 is not desired. A first approach in this case would be to improve the coupling efficiency even further, thereby utilizing the cofactors more efficiently and probably also increase the stability of the P450 by minimizing the production of reactive oxygen species. The stability of the P450 can be seen to be a major issue in this study and perhaps one of the most challenging aspects of whole cell catalysis. Protein engineering is the most promising alternative to increase the stability of the enzyme (Cirino and Arnold 2003; Salazar et al. 2003).

Characterization and identification of the limitations of a biocatalytic process is only the first step towards development of an economical feasible process. The performance also needs to be translated into quantitative targets and from there required improvements on the biocatalytic parameters identified. The economic targets, from Chapter 2, are presented in Figure 3.12. Regardless of the mode of operation, high final product concentrations (more than 20 g/L) are required to avoid high DSP cost. A high reaction yield, above 90 %, is also irrespective mode of operation. For a resting cell process, requirements on a biocatalyst yield of 10 g/g cdw and for a growing cell process the identified requirements on space-time yield is 2 g/L/h. In Figure 3.12 data from the first 8 h of a 10 mM batch biotransformation (results also shown in Figure 3.3) is represented in relation to the targets discussed above. With the introduction of solid substrate, the final product concentration could be increased 179 % (from 0.43 g/L to 1.2 g/L) compared to the control, where the substrate was pre-dissolved in DMSO using the same batch of cells. However, the biocatalytic performance of

different batches of cells was shown to vary and the economic calculations below are based on the process presented in Figure 3.3, where the substrate was supplied pre-dissolved in DMSO.

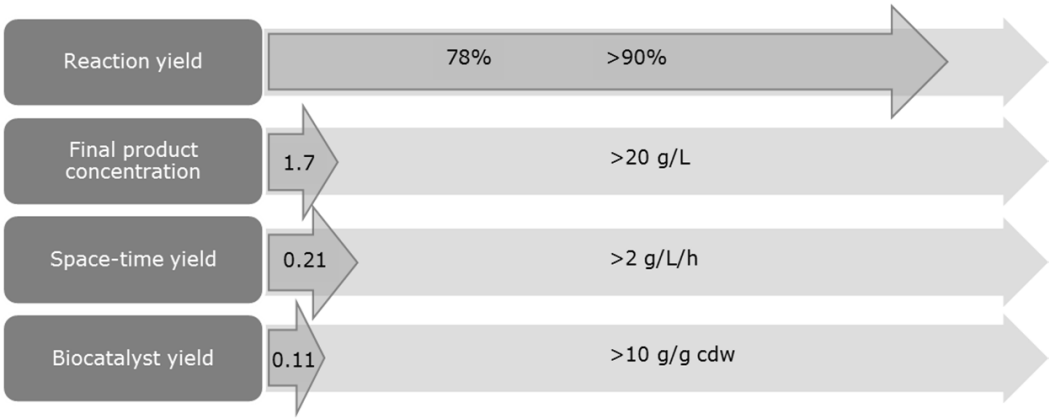


Figure 3.12 Metrics reached after 8 h in a batch process using 10 mM substrate are presented along with quantitative targets for an economically feasible process (Lundemo and Woodley 2015).

3.5 Recommendations for future development

In order to fulfill the required targets presented in Figure 3.12 several aspects of the biocatalytic reaction will require improvement. The reaction yield is close to reaching the target and when concentrations of substrate and catalyst are optimized it will likely move even closer.

The final product concentration can be improved by increased catalyst stability, increased catalyst concentration or by increased specific activity of the biocatalyst. As seen in Figure 3.12, a total improvement of 12x of these parameters would be required to reach the target. An increased stability of 3x to 24 h instead of 8 h in combination with 2x increase in both catalyst concentration and specific activity would lead to the target final product concentration of 20 g/L.

The space-time yield can be applied in a resting cell process to reflect the equipment occupancy even though the metric is of more importance for a growing cell process. In a resting cell process the space-time yield can be improved by increasing catalyst concentration and by increased specific activity. Unfortunately, a doubled catalyst concentration and 2x increased specific activity would not be

sufficient to reach the set target, the specific activity would have to be improved 5x instead. This could be addressed by protein engineering but the cofactor regeneration will probably be a challenge. As demonstrated in this study, the cofactor regeneration is a limiting factor for this resting cell process. The capacity of the cell needs to be utilized efficiently, by optimized enzyme expression and high coupling efficiency to minimize unproductive consumption of cofactor.

Even with the summarized improvements above the biocatalyst yield only reaches 1.65 g/g cdw, indicating that the catalyst in this case would have to be recycled 6 times in order to reach the target. This will most likely be challenging since more than 50 % of the correctly folded P450 is lost after 4 h of the reaction and indicates that the P450 process is preferably run using growing cells as operating mode.

The use of solid substrate is a promising approach to avoid the need for immiscible solvent that potentially can damage the catalyst and at the same time would cause the product to precipitate at higher concentrations. In research laboratories this might be a practical issue for analysis but in the final process it enables simple separation of the product from the broth.

3.6 Conclusions

Limitations for the resting cell P450 process of ω-hydroxylation of dodecanoic acid, catalyzed by CYP153A fused to the reductase domain of CYP102A1 expressed in *E. coli*, was demonstrated as a first step towards process development. Insufficient stability of the catalyst is the main hurdle for an economically feasible process. Cofactor regeneration and substrate and product inhibition also needs to be addressed, as well as demands on increased specific activity of the cell. Application of solid substrate is a promising approach to address the low stability of the biocatalyst as well as substrate and product inhibition by the avoidance of co-solvent, thereby lowering the amount of soluble substrate and product.

4 Case study II: CYP106A2 expressed in *Bacillus megaterium* catalyzing 15β-hydroxylation of cyproterone acetate

The model system chosen for the second case study represents a natural P450 expressing host, industrially established, and a multicomponent P450. The overexpressed P450, CYP106A2 is naturally expressed in *Bacillus megaterium* ATCC 13368 and was in this study overexpressed in *Bacillus megaterium* MS941, without overexpressing the still unknown redox partners. CYP106A2 has been characterized in Professor Rita Bernhardt's lab, Saarland University, Germany who also kindly provided the whole cell biocatalyst.

CYP106A2 from *Bacillus megaterium* ATCC 13368 was first identified as a regio- and stereoselective 15β-hydroxylase of 3-oxo-Δ4-steroids. Recently, it was shown that besides 3-oxo-Δ4-steroids, 3-hydroxy-Δ5-steroids as well as di-and triterpenes can also serve as substrates for this biocatalyst. It is highly selective towards the 15β position, but the 6β, 7α/β, 9α, 11α and 15α positions have also been described as targets for hydroxylation. Based on the broad substrate spectrum and hydroxylating capacity, it is an excellent candidate for the production of human drug metabolites and drug precursors.

In this work, the conversion of a synthetic testosterone derivative, cyproterone acetate, by CYP106A2 under *in vitro* and *in vivo* conditions was demonstrated. Using a *Bacillus megaterium* whole-cell system overexpressing CYP106A2, sufficient amounts of product for structure elucidation by nuclear magnetic resonance spectroscopy were obtained. The product was characterized as 15β-hydroxycyproterone acetate, the main human metabolite. Since the product is of pharmaceutical interest, the aim was to intensify the process by increasing the substrate concentration and to scale up the reaction from shake flasks to bioreactors to demonstrate an efficient, yet green and cost-effective production. Using a bench-top bioreactor and the recombinant *Bacillus megaterium* system, both a fermentation and a transformation process were successfully implemented. To improve the yield and product titers for future industrial application, the main bottlenecks of the reaction were addressed.

This chapter is based in large part on our paper submitted to *Microbial Cell Factories* (Paper III).

4.1 Introduction

P450s represent a suitable alternative over chemical synthesis, especially in the hydroxylation of steroidal pharmaceuticals, where the chemical methods are either time- and labor-intensive, expensive and complex or non-existent (Donova and Egorova 2012). Steroid-based drugs are one of the largest groups of marketed pharmaceuticals (Bureik and Bernhardt 2007). There are about 300 approved steroid drugs to date and their number is constantly growing due to the production of diversely functionalized steroid cores, resulting in often altered therapeutic activity (Tong and Dong 2009). Thus, steroid-hydroxylating P450s could provide an alternative for the production of drug precursors and human drug metabolites.

The bacterial P450, CYP106A2 from *Bacillus megaterium* (*B. megaterium*) ATCC 13368, is one of the few cloned bacterial steroid hydroxylases that has been studied in detail and was announced to be a suitable biocatalyst for the production of hydroxysteroids (Zehentgruber et al. 2010a). CYP106A2, also known as 15β-hydroxylase, converts mainly 3-oxo-Δ4-steroids (Lisurek et al. 2004; Berg et al. 1976; Kiss et al. 2014) although recent studies have shown that it can perform di- and triterpenoid hydroxylation (Bleif et al. 2012; Bleif et al. 2011; Schmitz et al. 2012) and the conversion of 3-hydroxy-Δ5-steroids (Schmitz et al. 2014). Moreover, as a result of on-going screening of a natural substrate library, the substrate range of this enzyme is constantly extended. However, the native substrate of CYP106A2, and thus its biological function, are still unknown. Its natural electron transfer protein is also unknown, yet the activity was successfully demonstrated using megaredoxin and megaredoxin reductase (Berg et al. 1976) and it is also supported by the bovine adrenal redox partners as well as by putidaredoxin and putidaredoxin reductase (Hannemann et al. 2006; Virus et al. 2006; Zehentgruber et al. 2010a).

In the past two decades CYP106A2 was profoundly investigated as a biocatalyst, applying the enzyme in whole-cell systems, efficiently using both *E. coli* (Hannemann et al. 2006; Zehentgruber et al. 2010a) and *B. megaterium* as expression hosts (Bleif et al. 2012; Bleif et al. 2011; Schmitz et al. 2012;

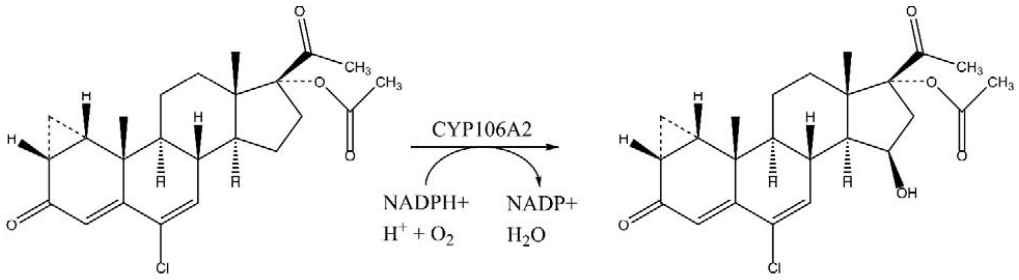
Schmitz et al. 2014). Whole-cell systems, in which the P450 is expressed by a microbial host, have the advantage of stabilizing the enzyme under process conditions and providing cofactor regeneration through cellular metabolism, avoiding the need for the expensive NADPH supply.

Since the transport of hydrophobic substances across the outer membrane of *E. coli* was found to be limiting (Zehentgruber et al. 2010a), the attention was shifted to the gram-positive *B. megaterium* as host. This spore-forming, mainly aerobic bacterium became a long-term participant of the biotechnological industry, due to the fact that even the wild-type strains are capable of producing high titers of proteins of industrial interest (Meinhardt et al. 1989; Barg et al. 2005). Further attractive characteristics include the ability to grow on a variety of carbon sources, the GRAS status, high plasmid stability and the lack of endotoxin and extracellular protease production. These characteristics make this organism highly favored for industrial practice (Vary et al. 2007).

The endogenous CYP106A2 system in *B. megaterium* ATCC 13368 was used for *in vivo* transformation of the diterpene abietic acid producing 12β-hydroxyabietic acid and 12α- hydroxyabietic acid (Bleif et al. 2011). As a next step, the CYP106A2 gene was overexpressed in combination with bovine adrenal redox partners in *B. megaterium* MS941 for the hydroxylation of 11-keto-β-boswellic acid in the 15α position (Bleif et al. 2012). Another triterpenoid, dipterocarpol, was also hydroxylated by CYP106A2 in *B. megaterium* ATCC 13368 resulting in six products at a 1 L scale (Schmitz et al. 2012). Recently reported, the regioselective hydroxylation of the 3-hydroxy-Δ5 steroid dehydroepiandrosterone (DHEA) was achieved by CYP106A2 expressed in the natural host *B. megaterium* ATCC 13368 and the recombinant *B. megaterium* MS941 (Schmitz et al. 2014).

In the present work, the conversion of a synthetic testosterone derivative, cyproterone acetate (CPA, 6-Chloro-1β,2β-dihydro-17-hydroxy-3'H-cyclopropa(1,2)-pregna-1,4,6-triene-3,20-dione acetate), was performed using a recombinant *B. megaterium* MS941 system overexpressing the CYP106A2 enzyme. The synthetic antiandrogen, CPA, was converted to 15β-hydroxy cyproterone acetate (15β-OH-CPA, 15β-hydroxy-6-Chloro-1β,2β-dihydro-17-hydroxy-3'H-cyclopropa(1,2)-pregna-1,4,6-triene-3,20-dione

acetate) (Scheme 4.1). CPA is a synthetic derivative of 17α-hydroxyprogesterone, an anti-androgenic compound with additional progestogen and antigonadotropic properties (Neumann and Toepert 1986; Frith and Phillipou 1985). It has antagonistic properties towards the androgen receptor, although it can also act as its partial agonist. It is generally used as a treatment for metastatic prostate cancer and for the control of libido in severe hypersexuality and/or sexual deviation in males, but it is also applied for the treatment of hirsutism and acne in female patients and in oral contraceptive pills. The main human metabolite of CPA in both plasma and urine is the 15β-OH-CPA. It shows only 10 % of the progestogenic potency of CPA but retains the anti-androgen activity (Frith and Phillipou 1985). These characteristics imply that the metabolite is potentially a better option for the treatment of androgen-induced problems, particularly in male patients. In 1982, the *B. megaterium* ATCC 13368 strain was already proposed for the bioconversion of 1α,2α-methylene steroids into their 15β-hydroxy derivatives, in order to produce new anti-androgenic steroids with minor progestogen activity (Petzoldt et al. 1982). According to our knowledge, 1 mg 15β-hydroxy metabolite costs 300 $, while 250 mg of the original compound costs 199 $ (Santa Cruz Biotechnology, http://www.scbt.com/, 2014). Although detailed information about the production is missing, the price difference suggests an expensive procedure.



Scheme 4.1 Cyproterone acetate conversion to its main human metabolite 15β-hydroxycyproterone acetate by CYP106A2.

In this study, process development of the CPA bioconversion in shake flasks and lab-scale bioreactors, was performed, thus providing an improved model for a greener yet cost-effective large-scale production of the 15β-hydroxy metabolite. The reaction was successfully carried out at 400 mL scale,

although to further improve the conversion rate the bottlenecks of the system were identified. Working with P450s applied in whole-cell systems, the following difficulties have already been recognized (Bernhardt and Urlacher 2014; Lundemo and Woodley 2015):

- NADP(H) depletion
- low substrate and product solubility
- problematic uptake of the substrate and efflux of the product
- substrate or product inhibition/toxicity.

To find the bottleneck of the current system, each point was addressed separately. The cofactor limitation was investigated by adding NADPH in excess. Issues with solubility, toxicity or inhibition, related to substrate or product were investigated. Subsequently substrate feeding strategies were evaluated in an attempt to overcome these effects. Moreover, the proposed transport restriction was addressed by using different membrane permeabilization methods (freeze-thawing, ultrasonication, acetone treatment). 2-hydroxypropyl-β-cyclodextrin (HP-β-CD) was also applied to improve the process performance since it was previously described to be successful as a solubilizing agent and was used for improved substrate transport across the cell membrane. By identifying the limitations of the system, the aim was to be able to improve the economic performance of the process by increasing the reaction yield at higher substrate concentrations.

4.2 Materials and methods

4.2.1 Reagents and chemicals

All used chemicals were from standard sources and of highest grade available. Solvents of analytical grade were used for HPLC, while solvents used for large-scale extraction were of reagent grade. CPA was obtained from BIOTREND Chemikalien GmbH, Köln, Germany (≥ 98% (HPLC)).

4.2.2 Bacterial strains and plasmids

The *in vivo* conversions were performed using a recombinant *B. megaterium* MS941 strain, a variant of DSM319, lacking the major extracellular protease gene *nprM* (Wittchen and Meinhardt 1995). The host organism was transformed with the plasmid pSMF2.1C (CYP106A2 gene introduced within the SpeI/MluI sites) by a polyethylene glycol-mediated technique using protoplasts (Bleif et al. 2012; Barg et al. 2005). As control, the wild type *B. megaterium* MS941 strain was used (lacking the pSMF2.1 plasmid, but naturally containing cytochrome P450 genes) to confirm that the reaction is catalyzed by the CYP106A2 enzyme. The wild type strain did not display any activity towards the substrate (data not shown) and no P450 was detected using CO difference spectroscopy (Omura and Sato 1964), indicating that the P450 expression and catalytic activity reported is assigned to the overexpressed CYP106A2.

4.2.3 Protein expression, purification and spectral characterization

The expression and purification of the CYP106A2 protein was performed as described previously (Simgen et al. 2000; Lisurek et al. 2004). For the reconstituted *in vitro* system, a truncated form of bovine adrenodoxin ($Adx_{4\text{-}108}$) was used in combination with bovine adrenodoxin-reductase (AdR), their expression and purification was completed as described elsewhere (Sagara et al. 1993; Uhlmann et al. 1992). The characteristics of the purified CYP106A2 were analyzed by UV-visible absorbance spectroscopy. The spectrum was recorded in a range of 200 to 700 nm with a double beam spectrophotometer (UV-1800, UV-2101 PC, Shimadzu Corporation, Kyoto, Japan) and analyzed constantly during the purification process to determine the Q value (A_{417}/A_{280}), which was in all cases above 1.5, suggesting a high amount of correctly folded, active P450s. The samples taken from the bacterial cultures during cultivation or conversion were spun down and the pellet kept frozen at -20 °C until measurement when the samples were resuspended in 100 mM potassium phosphate buffer, pH 7.4. The concentration of the purified protein and the protein expressed in the whole-cell system was determined by CO difference spectroscopy according to the method of Omura and Sato (Omura and Sato 1964), using an extinction coefficient of 91 mM^{-1} cm^{-1}.

4.2.4 Substrate binding assay

The substrate binding spectrum was investigated using a double-beam spectrophotometer (UV-2101PC, Shimadzu, Japan) and tandem quartz cuvettes. The analysis took place in 800 µL total volume. In one chamber, a cuvette containing 10 µM solution of CYP106A2 in 50 mM potassium phosphate buffer, pH 7.4 was placed. In the other chamber a cuvette filled with buffer was used as reference. CPA was dissolved in DMSO at a stock concentration of 10 mM. The enzyme was titrated with the substrate in a concentration range of 5 to 150 µM. After each titration step the spectrum was recorded in a range of 350 to 500 nm.

4.2.5 *In vitro* conversions and enzyme kinetics

The *in vitro* conversion of CPA was carried out with a reconstituted system in a final volume of 250 µL at 30 °C for 30 min in 50 mM potassium phosphate buffer (pH 7.4), containing 20 % (v/v) glycerol. The reconstituted system contained bovine AdR (1 µM), a truncated form of $Adx_{4–108}$ (10 µM), CYP106A2 (0.5 µM), a NADPH-regenerating system [MgCl2 (1 mM), glucose-6-phosphate (5 mM), glucose-6-phosphate dehydrogenase (1 U), and NADPH (0.1 µM)] and CPA (200 µM). The reaction was started by adding NADPH (1 mM) and incubated at 30 °C. The assay was stopped by the addition of 250 µL of ethyl acetate, mixed vigorously, and extracted twice. The combined organic phases were evaporated and the residues were dissolved in the HPLC mobile phase (60:40% ACN:H_2O) and subjected to analysis.

Reaction kinetics of CPA and CYP106A2 were performed for 2 min as described above, using bovine AdR (0.5 µM), bovine $Adx_{4–108}$ (5 µM) and CYP106A2 (0.25 µM). The substrate concentration was varied from 20 to 250 µM. Kinetic parameters were determined by plotting the initial reaction rate (nmol product/nmol P450/min) against the substrate concentration (µM). Each reaction was performed six times, the data represents the mean of these independent results. Data were fitted by hyperbolic regression with the help of Origin (OriginLab Corporation, Massachusetts, USA). The substrate inhibition studies were performed using a 20 mM CPA stock solution dissolved in DMSO and added in a final concentration of 200 to 1200 µM. Studying the product inhibition, the substrate was added in a final concentration of 400 µM, while the purified product concentration ranged from 0 to

1200 µM. The reactions took place for 60 minutes, then the samples were extracted and subjected to HPLC analysis as described above.

4.2.6 Heterologous expression in shake flasks

Pre-cultures were inoculated from a -80 °C glycerol stock, using 25 mL complex TB medium (24 g/l yeast extract, 12 g/l soytone, 2.31 g/l KH_2PO_4 and 12.5 g/l K_2HPO_4) supplemented with 10 mg/L tetracycline and incubated overnight, at 150 rpm, 30 °C. The main culture, containing 250 mL complex medium (supplemented with the corresponding amount of tetracycline) was inoculated with 1 % of the culture volume from the pre-culture. The main culture was incubated until an OD_{578} of 0.5, when 5 g/L xylose solution was added to induce expression. After 24 h expression, the cells were harvested (4500 x g , 4 °C, 15 min), the cell pellet was washed and resuspended in 100 mM potassium phosphate buffer (pH 7.4).

4.2.7 Heterologous expression at fermenter scale

The fermentation process was adapted from Korneli and coworkers (Korneli et al. 2012) . A -80 °C glycerol stock was used to inoculate the first pre-culture with LB medium which was used to inoculate a second pre-culture with batch medium (3.52 g/L KH_2PO_4, 6.62 g/L $Na_2HPO_4*2H_2O$, 0.3 g/L $MgSO_4*7H_2O$, 25 g/L $(NH_4)2SO_4$, 1 g/L yeast extract and trace elements as described (Korneli et al. 2012)). The first pre-culture was incubated for 8 h in a 100 mL shake flask with 10 mL LB medium at 150 rpm, 37 °C. 100 mL batch medium supplemented with 5 g/L fructose was used for the cultivation of the second pre-culture in a 1 L flask, inoculated from the first pre-culture to an OD_{600} of 0.1. After 12 h of cultivation, it served as inoculum for the fermenter. The batch medium in the fermenter was supplemented with 15 g/L fructose. The feed medium consisted of 150 g/L fructose, 5 g/L D-xylose, 9.9 g/L KH_2PO_4, 14.98 g/L Na_2HPO_4, 0.3 g/L $MgSO_4*7H_2O$, 25 g/L $(NH_4)_2SO_4$. All media preparations were supplemented with 10 mg/L tetracycline. The fed-batch fermentation process was carried out in a 1 L Infors Multifors fermentation vessel (Infors HT, Bottmingen, Switzerland). Initial conditions were set to 37 °C, pH 7.0, aeration 0.5 Lpm, pO_2 setpoint 20 % controlled by stirrer. The fermenter was inoculated to a final OD_{600} of 0.7 and induced at an OD_{600} of 10 with 5 g/L xylose. The induction at a higher OD_{600} in the fermenter, compared to shake flask, is due to the higher possible final cell density

utilizing a fed-batch process. At the time of induction, the temperature was decreased to 30 °C, aeration was increased to 1 Lpm and a linear feed was initiated. The pH was controlled during the process with 5 M NaOH and 1 M H_3PO_4. Cells were harvested by centrifugation and washed by resuspension in 100 mM potassium phosphate buffer, pH 7.4. 20 mM fructose was added to the cells for direct use in biotransformation or stored at 4 °C, gently shaking, at a cell density of 200 g/L WCW.

4.2.8 Bioconversion in shake flasks

The small-scale conversion of CPA was performed with resting cells in a 15 mL culture volume using 100 mL baffled shake flasks. The catalyst concentration was 100 g/L WCW, unless otherwise stated. To obtain sufficient amount of product (mg) for NMR structure characterization, the conversion was scaled up to 50 mL, using 300 mL baffled shake flasks. CPA was added in 200 µM final concentration from a 20 mM DMSO stock solution. The use of DMSO did not exceed 2 % of the culture volume. Every 4 h, 2 M fructose solution was added as carbon source in a final concentration of 20 mM. 250 µL samples were taken at indicated time points to monitor the conversion. The samples were extracted twice using 250 µL ethyl acetate, the organic phases collected and evaporated to dryness for the subsequent analysis by reverse-phase HPLC. In the case of product isolation, the reaction was stopped and the steroids were extracted twice by ethyl acetate. The organic phase was dried over anhydrous $MgSO_4$ and concentrated to dryness in a rotavapor (Büchi R-114). The yellowish residue was dissolved in the mobile phase of the HPLC and filtered through a sterile syringe filter (Rotilabo syringe filter, 0.22 µm, Carl Roth GmbH, Karlsruhe, Germany). The product purification was completed by preparative HPLC, according to its retention time. The collected fractions were evaporated to dryness and analyzed by NMR spectroscopy. The *in vivo* substrate and product inhibition was studied in shake flasks, using resting cells, as described for the *in vitro* studies.

4.2.9 HPLC analysis

The HPLC analysis was performed either on a Jasco system consisting of a Pu-980 HPLC pump, an AS-950 sampler, a UV-975 UV/Vis detector, and an LG-980–02 gradient unit (Jasco, Gross-Umstadt, Germany) or on a Dionex UltiMate 3000 HPLC equipped with a Photodiode Array Detector (Dionex, Thermo Scientific). A reversed-phase ec MN Nucleodor C_{18} (3 µM, 4.0x125 mm) column (Macherey-

Nagel, Betlehem, PA, USA) was used and kept at an oven temperature of 40 °C. An isocratic gradient of acetonitrile:water in a ratio of 60:40 was applied using a flow rate of 0.8 mL/min. UV detection of the substrate and product was accomplished at 282 nm. Product isolation was performed using preparative reversed-phase HPLC (ec MN Nucleodur C18 VP (5 µM, 8x250 mm), Macherey-Nagel, Betlehem, PA, USA) with a flow rate of 2.5 mL/min. The results are presented as conversion %, calculated from the product area divided by the sum of substrate and product areas. Regarding the product inhibition experiments, when product was added initially to the system, the data is presented as relative conversion (subtracting the initially added product) or calculated back to concentrations from conversions.

4.2.10 NMR characterization of the main metabolite

The NMR spectrum was recorded in $CDCl_3$ with a Bruker DRX 500 NMR spectrometer at 300 K. The chemical shifts were relative to TMS using the standard δ notation in parts per million. The 1D NMR (^1H and ^{13}C NMR, DEPT135) and the 2D NMR spectra (gs-HH-COSY, gs-NOESY, gs-HSQCED, and gs-HMBC) were recorded using the BRUKER pulse program library. All assignments were based on extensive NMR spectral evidence.

The main product (P1) was identified as 15β-hydroxy cyproterone acetate (15β-OH-CPA) (3.4 mg). In comparison to cyproterone acetate the NMR spectra of its conversion product showed signals for an additional secondary hydroxyl group (δ_H 4.50 ddd, J=7.5, 6.0 and 2.0 Hz; δ_C 68.86). Its position at C-15 could be deduced by vicinal couplings of the methine proton with H-14 (δ_H 1.93 dd, J=12.0 and 6.0 Hz), H-16α (δ_H 2.47 dd, J=16.8 and 7.5 Hz) and H-16β (δ_H 3.03 dd, J=16.8 and 2.0 Hz) in the HHCOSY and with C-13 (δ_C 47.00) and C-17 (δ_C 96.11) in HMBC. The β orientation of the hydroxyl was obvious by the NOESY effects of H-15 to H-16α and to H-9 (δ_H 1.53 m) and H-14, both in α position. In addition, the coupling constants found for H-15α resembled those for other closely related steroids, e.g. 15β-hydroxy-11-deoxycortisol (Kiss et al. 2014). Selected ^1H NMR data of 15β-hydroxy cyproterone acetate could be found in the literature (Bhargava et al. 1977) and matched with our values. ^1H NMR ($CDCl_3$, 500 MHz): δ 0.89 ddd (6.3 and 2x4.7 Hz, cPr-Ha), 1.01 s (3xH-18), 1.27 s (3xH-19), 1.29 ddd (9.0, 7.8 and 4.7 Hz, cPr-Hb), 1.53 m (H-9), 1.62 m (2H, H-11β and H-12β), 1.74 ddd

(2x7.8 and 6.3 Hz, H-1), 1.93 dd (12.0 and 6.0 Hz, H-14), 1.98 m (H-11α), 2.03 m (H-2), 2.04 m (H-12α), 2.09 s (3x OCOC*H*$_3$), 2.10 s (3xH-21), 2.47 dd (16.8 and 7.5 Hz, H-16α), 2.73 ddd (2x12.0 and 2.3 Hz, H-8), 3.03 dd (16.8 and 2.0 Hz, H-16β), 4.50 ddd (7.5, 6.0 and 2.0 Hz, H-15), 6.20 brs (H-4), 6.44 d (2.3 Hz, H-7). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 12.30 (CH_2, cPr), 16.95 (CH_3, C-18), 20.74 (CH_2, C-11), 21.11 (C, OCO*C*H$_3$), 22.86 (CH_3, C-19), 25.24 (CH, C-2), 26.12 (CH, C-1), 26.35 (CH_3, C-21), 31.96 (CH_2, C-12), 34.44 (CH, C-8), 38.84 (C, C-10), 43.13 (CH_2, C-16), 47.00 (C, C-13), 47.98 (CH, C-9), 52.80 (CH, C-14), 68.86 (CH, C-15), 96.11 (C, C-17), 120.47 (CH, C-4), 130.45 (C, C-6), 136.30 (CH, C-7), 152.32 (C, C-5), 170.51 (C, O*C*OCH$_3$), 197.98 (C, C-3), 202.84 (C, C-20).

4.2.11 Transport and cofactor dependence

Transport limitation was examined by different cell membrane permeabilization methods prior to the biocatalytic reaction. Both mechanical and chemical methods were applied (freeze-thawing, acetone treatment and ultra-sonication). Frozen cells were spun down and the pellet was kept at -20 °C overnight. Acetone-treated cells were incubated with 5 % acetone for 2 minutes while vortexing, mechanical disruption was performed by sonication for 2 minutes (amplitude 60 %, 0.5 s cycles) (UP400 S, Hielscher Ultrasonic GmbH, Teltow, Germany). Following the permeabilization treatment (acetone and sonication) cells were spun down and the pellet resuspended in 100 mM potassium phosphate buffer pH 7.4. Cofactor was added to the untreated control samples and to the permeabilized ones, once and twice stoichiometric amounts relative to substrate concentration.

4.2.12 Storage stability

To examine the storage stability of the whole-cell catalyst, the transformation was performed with resting cells after 1, 3 and 7 days of storage. The cells were stored at 4 °C, gently shaking at a cell density of 200 g/L WCW in 100 mM potassium phosphate buffer, pH 7.4. 20 mM fructose was added as carbon source at the time of harvest, after 1 and 3 days of storage and also at the start of the reactions.

4.2.13 Cyclodextrin

As an alternative substrate feeding strategy, the substrate was pre-dissolved in a 45 % (w/v) solution of HP-β-CD in sterile-filtered MilliQ water and stirred overnight using a magnetic stirrer.

4.2.14 Optical density and cell dry weight determination

To estimate the cell concentration, the optical density at 600 nm (OD_{600}) was monitored and the gravimetric dry cell weight (g/L DCW) was determined. Samples collected for dry cell weight measurement were spun down, the supernatant was discarded and the pellet was kept at -20 °C until further analysis. Thereafter the pellets were thawed and resuspended in the original sample volume using 100 mM potassium phosphate buffer, pH 7.4. Dry cell weight was measured in triplicates by filtering the samples through a pre-weighed 0.22 µm PES membrane filter (Frisenette, Knebel, Denmark) applying vacuum. The filters were washed with buffer, dried in a microwave oven and weighed after equilibrating to room temperature in a desiccator.

4.2.15 Bioconversion in bioreactor

Biocatalysis in bioreactors was performed in the same vessels as the fermentation (Infors Multifors, Infors HT, Bottmingen, Switzerland) in a working volume of 400 ml. Set points applied were: 30 °C, aeration 1 Lpm, pO_2 30 % controlled by agitation, pH 7.2 controlled with 5 M NaOH and 1M H_3PO_4. 2 M stock solution of fructose was added at time point 0, 4 and 8 h in a final concentration of 20 mM. CPA was dissolved in DMSO and added in a final concentration of 1 mM, the DMSO content not exceeding 2 % of the total volume. For an accurate comparison of growing and resting cells half of the fermentation volume was removed after 16 h and this fraction was harvested by centrifugation, washed and resuspended in 100 mM potassium phosphate buffer, pH 7.4. Resuspended cells were transferred to a bioreactor, simultaneously to the still growing cells. Fructose and xylose were continuously fed, for both resting and growing cells, at half the volumetric rate compared to the fermentation due to half the volume.

4.3 Results

4.3.1 Purification and spectral characterization of CYP106A2

The CYP106A2 protein was expressed and purified using a recombinant *E. coli* C43 (DE3) strain. The UV-Vis absorbance spectra recorded from 250 to 700 nm showed the characteristic absorbance peaks at 356, 417, 534, 567 nm in the oxidized form. In the case of the reduced and carbon monoxide-bound form, the peak at 450 nm was observed, with no peak indicating inactive P450 at 420 nm.

4.3.2 *In vitro* conversion, reaction kinetics and inhibition studies

Using difference spectroscopy, the binding of CPA to CYP106A2's active site was studied *in vitro*. CPA did not induce any spectral shift, indicating that the steroid does not contribute to the replacement of the axial water molecule, hindering the determination of the dissociation constant. The catalytic activity of CYP106A2 towards CPA was first tested *in vitro*. The activity was reconstituted using bovine adrenal redox partners ($Adx_{4\text{-}108}$ and AdR) proven to be highly efficient electron suppliers for CYP106A2 (Ewen et al. 2012; Virus et al. 2006). The CYP106A2-dependent conversion of CPA was analyzed by high-performance liquid chromatography (HPLC) and resulted in one main product. Using 0.5 µM CYP106A2 and 400 µM substrate, the conversion reached 48.2 ± 2.8 % in 60 minutes (Fig. 4.1).

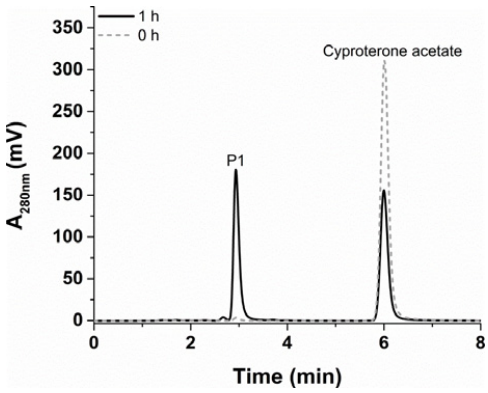


Figure 4.1 HPLC chromatogram of the *in vitro* conversion of cyproterone acetate by CYP106A2. The reaction was performed in 50 mM potassium phosphate buffer containing 20 % glycerol (pH 7.4) at 30 °C, using 0.5 µM CYP106A2, 10 µM $Adx_{4\text{-}108}$ and 1 µM AdR. The reaction was stopped and extracted twice by ethyl acetate directly after the addition of the substrate (grey dotted line, 0h) and after 1 h (black line, 1 h). Cyproterone acetate (400 µM) was converted to one main product (P1).

The *in vitro* conversions were also performed with increasing substrate concentrations (50 µM - 1.2 mM) to study the potential inhibitory effect of the substrate. Using 200 µM or higher substrate concentrations, the product concentration never exceeded 200 µM. These results suggest that the enzyme is inhibited above a certain product concentration, regardless of the amount of substrate, since the reaction stops after 150 to 200 µM product was formed (Fig. 4.2).

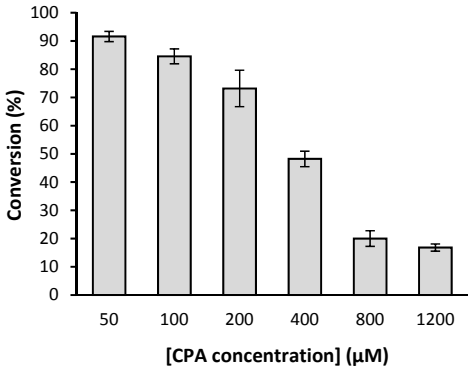


Figure 4.2 *In vitro* conversion of cyproterone acetate using increasing substrate concentrations. The reaction was performed using substrate concentrations, in a range from 50 µM to 1.2 mM for 60 min. Each bar represents the mean value of three independent measurements, with the corresponding standard deviation indicated by the error bars.

As a next step, the Michaelis-Menten parameters for the CYP106A2-dependent CPA conversion were determined. The catalytic activity of the CPA conversion showed a V_{max} of 61.65 ± 2.56 nmol product per nmol CYP106A2 per minute and a K_m of 103.14 ± 11.99 µM (Fig. 4.3).

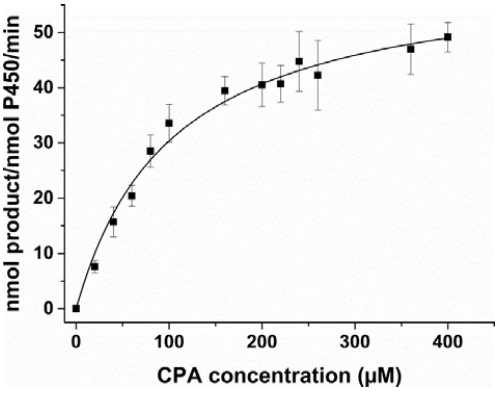


Figure 4.3 Determination of the kinetic parameters for the cyproterone acetate conversion catalyzed by CYP106A2. The reaction kinetics were performed in 50 mM potassium-phosphate buffer containing 20 % glycerol (pH 7.4) at 30 °C for 2 min using 0.5 µM CYP106A2, 10 µM $Adx_{4\text{-}108}$ and 1 µM AdR. Cyproterone acetate was used in a concentration range from 0 to 400 µM. The data shown are the result of four independent measurements ($R^2 > 0.98$).

To investigate potential product inhibition, *in vitro* product inhibition experiments were performed using a fixed amount of substrate with increasing initial product concentrations (purified by preparative HPLC). The results confirmed the assumption that the product used in 200 µM or higher concentrations strongly inhibits the reaction. Using an initial 15β-OH-CPA concentration of 800 µM or above, less than half the conversion could be observed (21 %) compared to the control sample (47 %) containing only the substrate (400 µM) (Fig. 4.4).

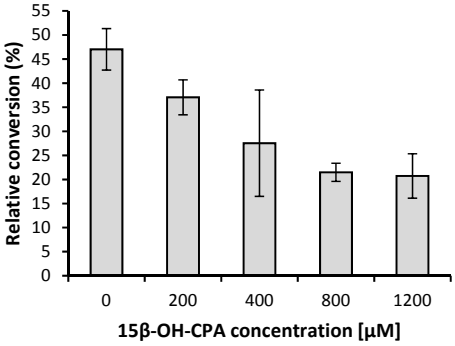


Figure 4.4 *In vitro* conversion of cyproterone acetate using increasing initial product concentrations. The reactions were performed using 400 µM substrate concentration and initial product concentrations ranging from 0 to 1200 µM for 60 min. The data represents the mean of three independent measurements with the corresponding standard deviation shown by the error bars.

4.3.3 *In vivo* conversion, product localization and catalyst reusability

Following the successful conversion of cyproterone acetate by the CYP106A2-overexpressing *B. megaterium* strain (Fig. 4.5), the product of the reaction was purified on preparative HPLC and its srtucture was identified by nuclear magnetic resonance (NMR) spectroscopy. The resulting main product (P1), 15β-OH-CPA, was used in the above-mentioned *in vitro* as well as in the *in vivo* product inhibition studies. The whole-cell catalyst was further characterized by examining the substrate and product localization. Both substrate and product were shown to be attached to the cell pellet fraction (data not shown). Adding more cells after 4 h of conversion did not improve the reaction yield, most likely since all remaining substrate was already inside or attached to the original cells (Fig. 4.6).

However, the addition of 3 times more cells (150 g/L wet cell weight (WCW)) and 2 times more substrate (1 mM) only doubled the product concentration thereby giving a lower biocatalyst yield.

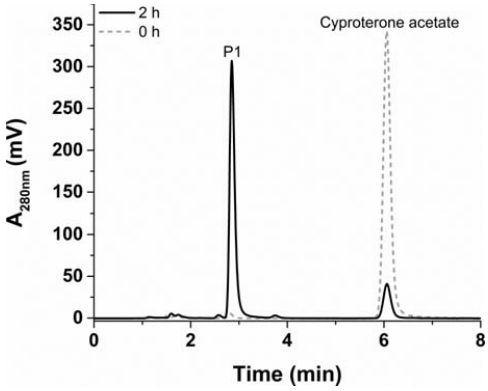


Figure 4.5 HPLC chromatogram of the cyproterone acetate conversion using *B. megaterium* MS941 overexpressing the CYP106A2 enzyme. The reaction was performed with resting cells in 100 mM potassium phosphate buffer (pH 7.4) at 30 °C, 150 rpm. Cyproterone acetate (400 µM) was added to the cells in DMSO solution (2 % v/v). Samples were collected directly at the point of substrate addition (grey dotted line) and after 2 h (black line). The substrate was converted to one main product (P1).

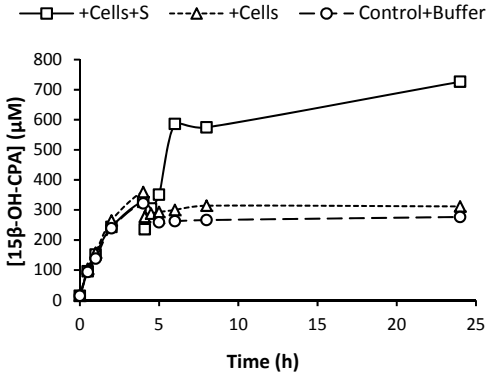


Figure 4.6 Effect of whole-cell catalyst addition, with and without additional substrate, on 15β-hydroxycyproterone acetate production. Reactions were performed in shake flasks with 1 mM cyproterone acetate and an initial catalyst concentration of 50 g/L WCW. After 4 h, additional 150 g/L WCW was added to the reaction represented with △, both catalyst and substrate (1 mM) were added to the reaction represented with □ and buffer was added to the control (○) to compensate for the change in volume.

In a further attempt to improve the biocatalyst yield, the reusability of the whole-cell system was investigated to decrease the cost contribution of the catalyst and increase the economic potential of a resting cell process. Removal of the product by solvent extraction between batches was explored. Washing with buffer did not have any effect on the product in the cell pellet fraction. Exposing the cells to the organic solvent ethyl acetate completely destroyed the activity of the cells. Furthermore, washing with decanol removed the product from the cells, but at the same time damaged the catalyst resulting in around 30 % relative conversion compared to the first batch (Fig. 4.7).

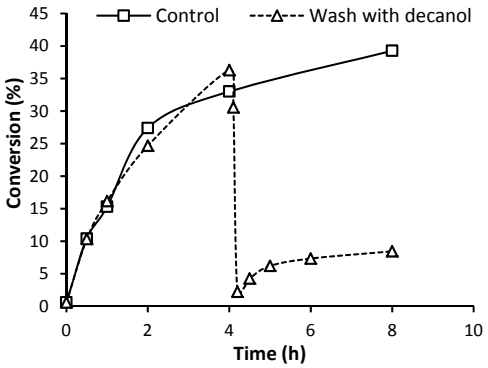


Figure 4.7 Cell recycling by product extraction using decanol. □ represents the control and △ the cells washed with decanol after 4 h. Reactions were performed with resting cells in shake flasks, using 1 mM cyproterone acetate and an initial catalyst concentration of 100 g/L WCW. 1 mM substrate was added when starting each reaction but only to the washed cells at 4 h.

4.3.4 Transport and cofactor dependence

The potential limitations of the whole-cell system in terms of substrate transport across the cell membrane and cofactor regeneration were ruled out as seen in Fig. 4.8. The reactions were performed with resting whole-cells in shake flasks and NADPH was added in 1 mM (stoichiometric to substrate) and 2 mM final concentrations. The influence of cofactor addition and the cell permeabilization methods (sonication, acetone treatment or freeze-thawing) on the reaction rate was investigated. The initial rates measured in these experiments did not show any significant difference (data not shown). In addition, Figure 4.8 shows that no significant difference was observed in the final product concentration either, regardless of permeabilization treatment or external addition of cofactor. The cofactor is assumed to pass the cell membrane and enter the cell, similarily to observations in *E. coli* where this was monitored by a decreasing absorbance of the supernatant at 340 nm. The results demonstrate that the cofactor regeneration of the host *B. megaterium* MS941 is sufficient to support the observed biocatalytic reaction rates and that the natural redox partners are sufficiently expressed to transport electrons from the cofactor to the active site of the overexpressed P450. The substrate transport that has been shown to limit a CYP106A2-catalyzed steroid transformation in *E. coli* (Zehentgruber et al. 2010a) was not limiting the reaction studied here in *B.*

megaterium, according to the tested permeabilization methods, emphasizing the suitability of this whole-cell catalyst.

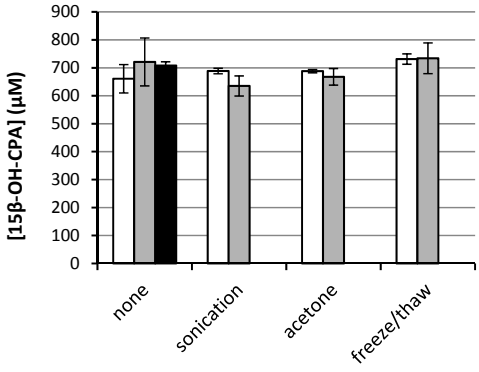


Figure 4.8 Effect of different cell permeabilization methods and cofactor addition on 15β-hydroxycyproterone acetate production. White bars represent the results without cofactor addition, grey bars have 1 mM NADPH and the black bar have 2 mM NADPH added. All reactions were run using 1 mM cyproterone acetate, 2 % DMSO and 100 g/L WCW cells from the same fermentation batch. Error bars are 1σ.

4.3.5 *In vivo* substrate and product inhibition

The influence of increasing substrate and product concentrations on the reaction performance was also investigated *in vivo* with the recombinant *B. megaterium* MS941 strain overexpressing CYP106A2. The substrate inhibition studies were performed within a concentration range of 50 μM to 1 mM. When using up to 200 μM substrate concentration, complete conversion of the substrate took place already within 2 h, while at higher substrate concentrations the conversion stopped at an approximate product concentration of 300 μM (70 % conversion in the case of 400 μM and 27 % in the case of 1000 μM CPA) (Fig. 4.9), showing a similar trend as the *in vitro* experiments.

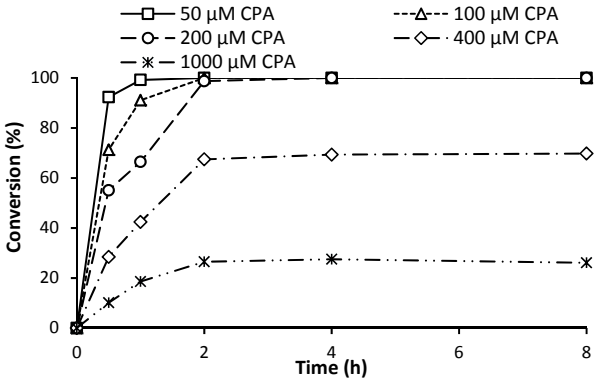


Figure 4.9 *In vivo* conversions performed in shake flasks with increasing substrate concentrations using resting cells. The reactions were performed in 100 mM potassium phosphate buffer (pH 7.4) at 30 °C, 150 rpm, with a substrate concentration ranging from 50 µM to 1 mM.

4.3.6 Product removal strategies

To remove the product and push the reaction equilibrium in the forward direction, thereby enhancing the reaction performance, the use of two water-immiscible solvents, diisononylphtalate ($C_{26}H_{42}O_4$) and hexadecane ($C_{16}H_{34}$) (selected to be compatible with the whole-cell catalyst and with oxygen requiring reactions) was investigated. However, solubilization of the substrate in these solvents, prior to addition to the aqueous phase containing the whole-cell catalyst, did not improve the reaction performance. The hydrophobicity of the substrate hinders the partitioning to the aqueous phase and thereby hampers the catalytic reaction (results not shown). The negative results could also be explained by analytical difficulties and problems with homogenous sampling in a solid-liquid 2-phase system.

Another approach to maintain a low concentration of the dissolved product in the aqueous phase is to avoid the use of a miscible co-solvent and instead apply feed of the solid substrate. This approach requires that the rate of solubilization of the substrate into the aqueous phase is faster than the reaction rate so that it does not limit the observed reaction. However, this method was not successful either, most likely due to the low solubility and rate of dissolution of the substrate.

A third approach was to pre-solubilize the substrate in an aqueous solution of HP-β-CD ($(C_6H_9O_5)_7(C_3H_7O)_{4.5}$), in order to take advantage of its multiple effects. Cyclodextrin (CD), especially the derivatized forms, have been shown to be useful in enhancing steroid conversions by e.g. increasing the cell-wall permeability, stimulating cell growth and efficiently solubilizing hydrophobic substrates (Donova and Egorova 2012). The complexation of β-CD with substrate and/or product also leads to lower amounts of free dissolved species and thereby lower inhibitory effects of either substrate or product on the catalyst, as suggested previously for steroid biotransformations (Roglic et al. 2007; Singer et al. 1991). Using CD-solubilized CPA, the transformations were performed first in shake flasks with 1 mM final concentration of the substrate. As a control, the conversion was also performed with the substrate dissolved in dimethyl sulfoxide (DMSO). The CPA was added from the 45 % CD solution, not exceeding 5 % of the reaction volume. During the conversion, 250 µl reaction samples were taken at the indicated time points and the product/substrate ratio was analyzed by HPLC. Despite the slow initial rate, a higher conversion was reached within 4 h using CD as solubilizing agent, compared to the control. After 24 h, the conversion with CD showed 38 ± 0.05 % product formation, while the control could only reach 27 ± 4.6 % (Fig 4.10). Given the improved conversion, the same strategy was applied in the bioreactor (Figure 4.11). 98 % conversion of 1 mM substrate was achieved on a 400 mL scale compared to a final conversion of 55 % for the control without CD.

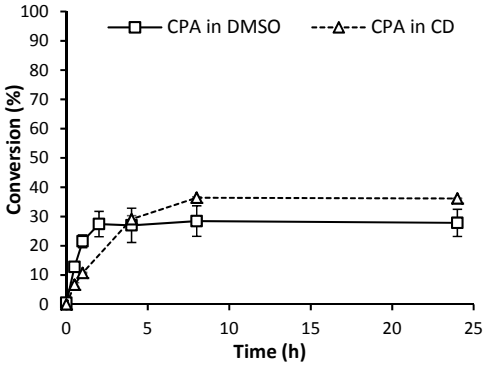


Figure 4.10 *In vivo* cyproterone acetate conversion in shake flasks, using 2-hydroxypropyl-β-cyclodextrin and DMSO for substrate solubilization. The reactions were carried out with resting cells, in 100 mM potassium phosphate buffer (pH 7.4) at 30 °C, 150 rpm. The substrate was pre-dissolved either in a 45 % CD solution mixed overnight (△) or in DMSO (□) with a final concentration of 1 mM.

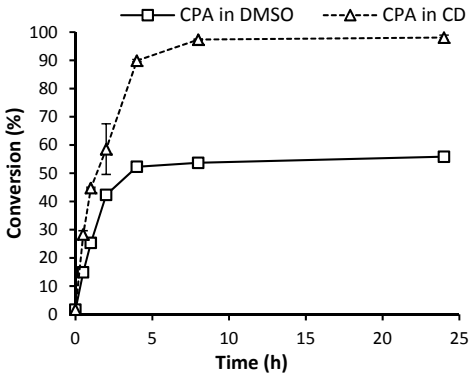


Figure 4.11 *In vivo* cyproterone acetate conversion in bioreactor, using 2-hydroxypropyl-β-cyclodextrin and DMSO for substrate solubilization. The reactions were carried out in 400 ml final volume with resting cells resuspended in 100 mM potassium phosphate buffer (pH 7.4) at 30 °C. The substrate was either pre-dissolved, in a 45 % CD solution mixed overnight (△) or in DMSO (□) with a final concentration of 1 mM.

4.3.7 Influence of reaction mixing

In this study, catalyst-dependent parameters, believed to be independent of the used scale, were investigated in shake flasks. Shake flask experiments are commonly used in research laboratories, providing a simple and fast tool to demonstrate the proof of concept. However, for process development and scale-up studies, a more controlled environment and a more easily scalable configuration is preferred. When comparing batch transformations between bioreactor and shake flasks under identical conditions, the former showed faster initial rates (Fig. 4.12). This result indicates that increased mixing enhances the reaction rate, most likely due to increased mass transfer of poorly water-soluble substrate but also due to the increased aeration. These results also suggest that for processes targeting scale-up and industrial implementation, process development should be performed with the intended final reactor configuration, in this case a stirred tank reactor instead of shake flasks. This especially concerns reactions involving species with low water solubility and gaseous components (e.g. oxygen).

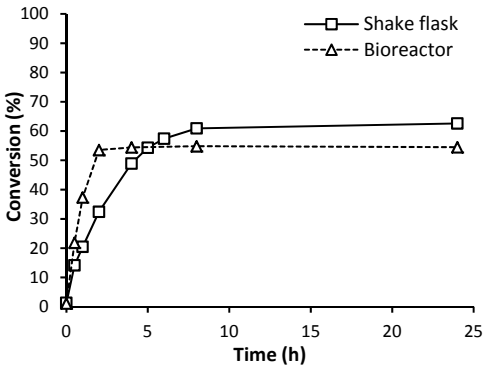


Figure 4.12 Comparison of 1 mM cyproterone acetate transformation in bioreactor and shake flask. The reactions were carried out using the same batch of cells, in a resting cell format (100 g/L WCW), and the substrate was added dissolved in DMSO. The initial rate of the reaction performed in the bioreactor (△) showed a higher initial rate compared to the reaction performed in shake flasks (□), although the reaction in the shake flask resulted in a higher final conversion.

4.3.8 Catalyst stability

The stability of a biocatalyst is crucial for the economic potential of a biocatalytic process. In this study we examined the storage stability, due to practical reasons, and more importantly, the stability under process conditions, under which the relevant information is collected. The dry cell weight and the correctly folded P450 were monitored during the process and it was found that the cells remained intact but the stability of the CYP106A2 was limited. One third of the correctly folded P450 is degraded after 4 h of the reaction and more than 50 % is lost after 24 h (Fig. 4.13).

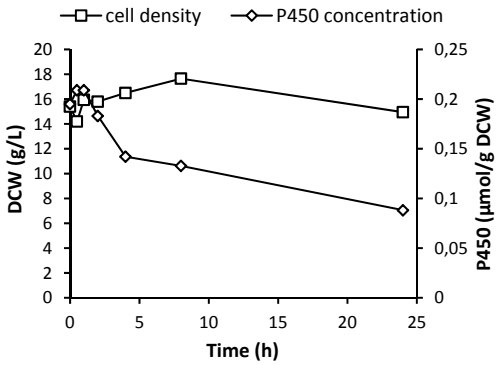


Figure 4.13 Stability of the whole-cell catalyst under process conditions. Reaction performed with resting cells in the bioreactor at 1 mM cyproterone acetate concentration and a cell density of 100 g/L WCW. Samples were collected at the indicated time points. The dry cell weight (□) and the P450 content (◇) were measured.

The storage stability of the catalyst was determined by running resting cell biotransformations with cells previously resuspended in buffer and stored gently shaken at 4 °C. No significant loss in reaction performance was found over 7 days of storage (data not shown). The stability of the catalyst could potentially also be affected by the mode of operation. However, no significant difference was seen between growing and resting cells in terms of conversion, whole-cell stability or P450 stability.

4.4 Discussion

Even though in the past decade significant progress was made to develop efficient biocatalysts for steroid transformations, there is still a great demand for greener and cost-efficient routes for valuable

steroid production (Donova and Egorova 2012). Microbial steroid transformations, using heterologously expressed cytochrome P450 enzymes are considered to be a promising approach, since the regio- and stereospecific hydroxylations are known to be challenging for synthetic steroid chemistry, suffering from low predictability and specificity (Bernhardt and Urlacher 2014). However, in most cases the reactions performed at analytical scale are not directly applicable for industrial practice. For an industrial process, the economic feasibility needs to be considered. For guidance, at the current stage of process development, targets for economic metrics such as space-time yield (for a growing cell process), final product concentration and biocatalyst yield (for a resting cell process) can be used. For high-value products or pharmaceuticals, such as the 15β-OH-CPA, target values for product concentration of 20 g/L, a space-time yield of 2 g/L/h (for a growing cell process) and a biocatalyst yield of 10 g product/g dry cell weight (DCW) (for a resting-cell process) can be used as reference, as identified in Chapter 2. These targets can only be achieved using higher substrate concentrations, and problems associated with higher concentrations such as low solubility of the substrate, potential substrate or product inhibition and toxicity most likely will arise.

Hannemann and co-workers converted 11-deoxycorticosterone to 15β-hydroxy-11-deoxycorticosterone using CYP106A2, functionally expressed with bovine adrenal redox partners in a growing whole cell *E. coli* host. According to our calculations, the molar substrate concentration applied in this study (0.5 mM) could result in a maximal final product concentration of 0.17 g/L. Furthermore, since the substrate was not fully converted after 24 h, the reported space-time yield of 0.33 g/L/d is misleading (Hannemann et al. 2006). However, the focus of the study was to design a whole cell catalyst and demonstrate the applicability of a screening assay. The whole-cell system was then further improved by the coexpression of a cofactor regeneration system and used for the 15β-hydroxylation of progesterone and testosterone (Zehentgruber et al. 2010a). This system was shown to be limited by substrate transport across the cell membrane and solubility of the substrate was shown to be crucial for the reaction performance. The productivity was reported to be 5.5 g/L/d using 0.5 mM initial substrate concentration and a lyophilized cell extract on a 20 mL scale. The reported productivity in this case is also misleading since it was extrapolated from a 30 min reaction, the 96 %

conversion can be translated to a final product concentration of 0.15 g/L. In the same study growing cells showed 25 % selectivity towards the 15β position, considerably lower than resting cells. More recently, DHEA was regioselectively hydroxylated to 7β-OH-DHEA by CYP106A2 expressed in *B. megaterium* (Schmitz et al. 2014). A benchmark 0.44 g/L total final product concentration was obtained upon addition of 1.5 mM substrate in a repeated batch mode, yielding 6 different products in 12 h, which is the highest reported concentration of the studies above. The selectivity towards the 7β-position was improved from 0.7 to 0.9 by changing host from *B. megaterium* strain ATCC 13368 to MS941. The bottlenecks of the reaction were discussed but never investigated.

In the current study, a systematic approach was taken to identify catalyst- and reaction-related limitations to enable an efficient process with defined targets based on economic guidance. By addressing the identified limitations we managed to improve the process performance. This is a novel approach compared to previous studies on steroid hydroxylation by CYP106A2. The regio- and stereoselective 15β-oxidation of CPA was demonstrated, in shake flasks and laboratory scale bioreactors. CYP106A2 was overexpressed in *B. megaterium* MS941 and proved to be a robust catalyst for the synthetic biotransformation of CPA. The *B. megaterium* host offers a protective environment for the enzyme, enhancing its stability, besides the cofactor regeneration provided by the cellular metabolism, which, according to our studies, was shown to be sufficient to not limit catalysis. The substrate transport across the membrane of the *B. megaterium* whole-cell catalyst turned out not to be a limiting factor either, as was described in the case of steroid hydroxylation by *E. coli* (Zehentgruber et al. 2010a). In contrast, the low overall solubility of the reactants, the limited stability of the P450, in combination with product inhibition, are suggested to be the main bottlenecks of this system.

The low solubility of substrate is a common challenge to many P450-catalyzed reactions. In the case of CPA the solubility is reported to be 0.64 mg/L (ChemSpider d) and 34 mg/L for the hydroxylated product (ChemSpider a). However, this should be put in perspective to the aimed final product concentration of 20 g/L. Low steroid solubility is usually solved by the application of a water

immiscible co-solvent but this approach has limitations at higher concentrations when the co-solvent can damage the biocatalyst (Laane et al. 1987). This can partly be circumvented by the application of CDs. These cyclic oligosaccharides are used in e.g. drug delivery (Davis and Brewster 2004) and have also been applied in P450-catalyzed reactions, mainly as a solubilizing agent and to improve substrate transport across the cell membrane, however at limited substrate concentrations (50 mg/L) (Roglic et al. 2007). The effects of HP-β-CD on steroid dehydrogenation of the gram-positive bacteria *Arthrobacter simplex* was investigated by Shen et al. (2014). Cells pretreated with HP-β-CD showed double the initial rate and reached final conversion 1 h faster compared to the non-treated cells, although in the end both reached the same concentration. This was shown to be the result of the cell membrane permeabilization by altered lipid and protein profiles of the membrane. CDs have also been applied in other fermentation and biotransformation processes to avoid toxic and inhibitory effects of the substrate or product, as summarized by Singh et al. (2002). In the current study, using HP-β-CD, 98 % conversion of 1 mM CPA regio- and stereoselectively within 8 h was achieved, resulting in 0.43 g/L product. This matches the literature benchmark for steroid conversion by CYP106A2 (Schmitz et al. 2014), yet the 8 h conversion time improves their space-time yield by 31 %. This new approach of bottleneck identification, takes P450-catalyzed reactions one step further towards higher product titers and economic viability. In our system, the time-dependent product inhibition was shown to limit final achievable product concentrations at higher substrate concentrations, thereby decreasing the reaction yield. By overcoming this problem, the main effect of CD was believed to be the complexation of the product, thereby pulling it out of the hydrophilic environment present in the cell and pushing the equilibrium towards the product formation.

4.5 Conclusions

Ultimately, with the help of HP-β-CD, a nearly complete conversion and a product formation of 0.43 g/L at a 400 mL scale was achieved, getting closer to industrial process requirements and a future large-scale application. However, in order to fully exploit the potential of the CD process, further optimization studies should be performed. Using CD to circumvent the identified bottlenecks of solubility and product inhibition, the stability of the P450 is still a challenge for an economically

feasible process. Considering that the stability of the enzyme will make cell recycling difficult, and that no significant differences could be found between a growing and resting cell process, the preferred operating mode for further process development would be growing cells. As stated above, the targets for an economically feasible growing cell process are a final product concentration of 20 g/L and a space-time yield of 2 g/L/h (Lundemo and Woodley 2015). Although a successful process development towards the suggested values by addressing the limitations was performed, there is still room for improvement.

5 Cost assessment of P450 catalyzed whole-cell processes

As a last part of the thesis, the knowledge gained from the already presented model systems and especially from the first (Chapter 3), where *E.coli* was applied as microbial host, was applied to a third model system with the main intention to perform an economic assessment by construction of a process model. The most explored P450, CYP102A1 from *Bacillus megaterium*, a natural fusion construct, was used as basis for this study. A double mutant, constructed by Michael Ringle, Lonza AG, Switzerland, shown to hydroxylate 4-ethylphenol to 4-ethylcatechol was expressed in the same *E. coli* strain as applied in Chapter 3 and the whole cell biocatalytic process was studied in 2.5 L fermenters in parallel to shake flasks.

Economical feasibility is of key importance for all production processes, regardless of their design and catalyst. Biocatalytic processes are known to be highly selective and in general environmentally friendly due to the mild conditions under which the processes can be performed. Here, an economical assessment based on a process model of a whole cell P450 catalyzed process is presented. The enzyme expression and enzyme total turnover have been varied with values taken from experiments performed on a 2.5 L scale as starting point. Furthermore, these whole cell processes can be performed in different operating modes, which have also been evaluated.

The aim of this study was to enable economic assessment of P450 catalyzed whole cell processes. The economic assessment can also be used to confirm the analysis made in Chapter 2. The work presented in this chapter is the result of an external stay at Lonza AG, Visp, Switzerland and based on Paper IV.

5.1 Introduction

Chemical process design has traditionally been based on economic viability utilizing models of different complexity. In recent years, the ecological (hazardous substances and environmental impact judged by e.g. a life cycle assessment (LCA)) and social (environmental, health and safety (EHS)) performance has also been included in the design (Ouattara et al. 2012; Albrecht et al. 2010).

Frameworks including process models at various stages of the process design can be used for screening of synthesis routes and process options with the information available at that development stage (Albrecht et al. 2010). This includes comparisons of e.g. a chemical synthesis route with biologically mediated synthesis (Adlercreutz et al. 2010; Johnston et al. 1987; Kuhn et al. 2010). In general, biotechnological processes have the advantage of being highly regio and stereo selective circumventing the need for protection and deprotection steps and the need for advanced methods in downstream processing (DSP). Furthermore, the cost distribution may differ between the chemical and biochemical processes and therefore assessments needs to be made on a case-by-case basis. E.g. dilute water streams and capital cost are perhaps the biggest challenges for biocatalytic processes whereas the raw material costs including the catalyst cost is dominating in a chemical process (Kuhn et al. 2010). In fermentation processes the dominating cost items have been described to be labor, capital cost and raw materials (Datar 1986; Harrison and Gibson 1984). The cost contribution of raw materials also depends on the type of product, generally increasing with decreasing product value and can be in the range of 70 % for bulk chemicals (Harrison and Gibson 1984). Regardless of the different benefits of the processes, economic profitability is the key parameter determining the fate of a process. All major chemical and pharmaceutical companies use various tools for process improvement and including tools for economic assessments (Bode et al. 2011; Harrison and Gibson 1984). However, standard costing tools for biocatalytic processes are not well established and the sensitivity of this type of information also hinders the development. In the scientific literature there is even more room for exploration of the field of economic and environmental process assessments, although it has been initiated (Lima-Ramos et al. 2014). An assessment is beneficial when scaling up and can also help to identify bottlenecks in a process and help to identify components that are the main cost contributors and needs to be optimized or improved (Harrison and Gibson 1984; Kuhn et al. 2010). At a basic research stage the cost for the required improvements can also be weighed against the development cost and likelihood of improving the parameter to the target value and thereby guide research efforts (Riet 1986). This has been done in general for fermentation processes, where the product of interest is produced during fermentation e.g. as secondary metabolite (Riet 1986).

In this study a process model is used to guide the development of whole cell P450 catalyzed reactions by correlating the final production cost with biological parameters such as enzyme expression and enzyme total turnover. Cytochrome P450 monooxygenases is a group of enzymes able to perform hydroxylation of non-activated hydrocarbons including aromatics (Bernhardt and Urlacher 2014; O'Reilly et al. 2011; Urlacher and Girhard 2012). However, only a few industrial P450 processes have been implemented so far. This can be explained by the many challenges in making P450 catalyzed processes economically feasible, in particular limited stability and low activity of the enzyme (Lundemo and Woodley 2015). Furthermore, the need for stoichiometric amounts of nicotinamide cofactor and redox partners transporting electrons from the cofactor to the active site of the monooxygenase urge the use of metabolically active whole cell catalysts. Metabolically active cells require that the cell is used for the catalytic reaction in close connection with an initial fermentation step. Two operating modes can be applied: growing cells where the biocatalytic reaction is performed simultaneously to the fermentation or resting cells where the cells are harvested after the initial fermentation by filtration or centrifugation and resuspended in buffer for the biocatalytic reaction step. In the latter case the biocatalytic step can be optimized to a greater extent compared to growing cells and also allows recycling of the catalyst, if the product localization and DSP permits. In this study an economical assessment of a P450 catalyzed process has been made based on a process model enabling construction of mass-balances over the entire process with the aim to put the biological parameters (protein expression and enzyme total turnover) in relation to the actual cost for production. Furthermore, the operating mode is evaluated as well.

5.2 Methodology

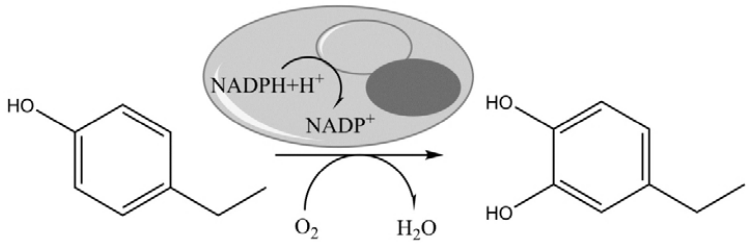
5.2.1 Approach

Process flow sheets were built in the process modeling and evaluation software SuperPro Designer (Intelligen Inc, Boston, MA) (SPD). The flowsheets were based on experimental data collected at a 2.5 L scale (Appendix 1) and scalability has been assumed. Based on the process model, including mass-balances, economic assessments of the process was made. The economic analysis was based on the unit production cost taken from the economic evaluation report generated in the software.

5.2.2 Model

The cost calculations have been done with the assumption of an existing multi-purpose plant. The quantities of the type of products produced by P450 catalyzed processes are not assumed to be large enough to make a dedicated plant suitable. The operating costs have been adjusted with a facility dependent cost based on equipment usage. The SPD default value of 100 $/h has been applied. Throughout the process, labor is calculated on the assumption that the process requires 1 man hour/hour. All procedures include a cleaning in place (CIP) procedure after each batch and the fermentation includes a sterilization in place (SIP) procedure prior to each batch. Inoculum preparation and waste treatment costs have not been included in the assessment.

The basis for the model has been a CYP102A1 double mutant expressed in *Escherichia coli* HMS174 catalyzing the hydroxylation of 4-ethylphenol to 4-ehtylcatechol (Scheme 5.1).



Scheme 5.1 Model reaction used as a base case in this study, hydroxylation of 4-ethylphenol to 4-ethylcatechol.

5.2.3 Base case: Resting cell process

A base case was set up using SuperPro Designer. The process is divided in three parts, fermentation followed by biocatalysis and finally DSP (Figure 5.1).

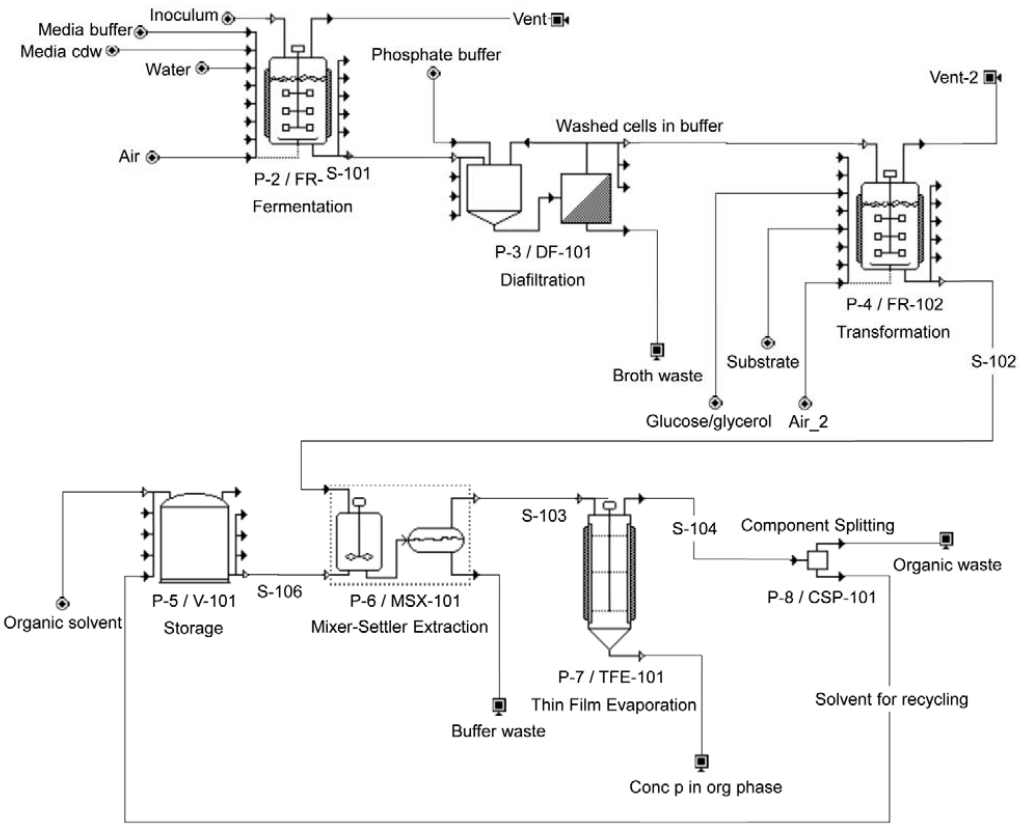


Figure 5.1 SuperPro Designer flowsheet of the resting cell process used for economic assessment.

The model is based on experimental data from the hydroxylation of 4-ethylphenol to 4-ethylcatechol by a CYP102A1 double mutant expressed in *E. coli* HMS174. The data for the fermentation and transformation were collected from experiments run on a 2.5 L scale (Appendix 1). The process is assumed to be run in a fermentation plant with two 50 m^3 fermenters and a microfiltration/diafiltration unit. The first two DSP operations are also assumed to be performed in the fermentation plant while further purification is expected to be performed in a multi-purpose chemical plant. This assumption is based on the potential to collect product streams from several fermentation batches and run further DSP in a semi-continuous mode (Riet 1986). The process model

and analyses up to a concentrated organic product stream can be applied to many P450 catalyzed processes, since the majority of the catalyzed products are hydrophobic and a concentration step is preferred in the initial DSP to minimize the volumes to be treated. From this point it is, however, more difficult to generalize and the last DSP steps, including purification, would vary depending on the product. Furthermore, a concentrated organic product stream is directly comparable to a corresponding stream from a potential competing chemical process for products where this would be the target of the cost assessment.

Other fixed process parameters have been summarized in Table 5.1 and are further discussed below.

Table 5.1 Fixed process parameters

Part of process	Parameter	State and motivation
Fermentation	Duration and final cell concentration.	P450 expression has been shown to be optimal at a growth rate of 0.1 h^{-1} and a feed phase lasting 4 generations.
Diafiltration	Area	131 m^2
	Duration	24 h
Biocatalysis	Cell density	Identical to fermentation to fully utilize the capacity of the fermenter where the biocatalytic step is performed.
	Duration	The stability of the P450 is assumed to limit the process to 8 h.
DSP	Product localization	Products assumed to be present in or attached to the cell membrane, cell separation therefore excluded. This assumption also excludes cell recycle.

5.2.3.1 Fermentation

The fermentation process was run as batch with semi-defined media followed by fed-batch with minimal media (Marisch et al. 2013). The batch phase was assumed to last for 12 h followed by a fed-batch phase with a fixed growth rate of 0.1 h^{-1} yielding a final cell density of 33 g cdw/L. The fermentation process was run in total for 40 h and for simplistic reasons this is shown as one process with one inlet media stream dependent on the volume and one stream dependent on the desired final biomass concentration. The duration of the fermentation process and biomass concentration has in this case not been varied.

5.2.3.2 Diafiltration

The cells are harvested and reaction media changed by diafiltration. Four volumes of 100 mM potassium phosphate buffer is used and the cell concentration is kept constant, although it potentially could be changed. Assumed filter area is 131 m^2.

5.2.3.3 Biocatalysis

The base case biocatalytic reaction is based on values from experiments performed at 2.5 L scale and presented in Appendix 1. Substrate is fed based on the P450 content in the inlet stream (1 g substrate/g P450 in the base case, corresponding to 10 mM final substrate concentration). The assumption that differs from the results in the supplementary information is that an improved reaction yield of 90 % is assumed. The substrate stream consists of 10 % substrate in DMSO and the transformation is performed during 8 h at 30 °C. The substrate, 4-ethylphenol, is estimated to a cost of one tenth of the catalogue price in SigmaAldrich (4.9 $/kg). The metabolic activity of the cells was retained by feeding carbon source (glucose/glycerol mixture) on a mass basis dependent on the biomass in the inlet stream to the transformation step (7 g stock mixture/g cdw).

5.2.3.4 Downstream processing

The downstream processing is the part of the process that will vary most dependent on the reaction system. In the base case the hydrophobic product is assumed to be present attached to the cell membrane and therefore a mixer-settler extraction is chosen, followed by thin film evaporation to concentrate the organic phase containing the product. This also implies that cell recycle has not been considered. The chosen DSP will result in a stream for further purification comparable with a stream from an alternative chemical synthesis route so that the two competing routes until this point can be compared.

The product will first be extracted using an equal volume organic solvent, in this case ethyl acetate (40 000 L/batch), in a mixer-settler extraction with a batch duration of 6 h. Extraction and evaporation data has been assumed using the properties naphthalene and naphthol found in the Designer database in SuperPro Designer. Partition coefficients have been assumed to be the same as the logP (octanol/water) due to missing experimental data for ethyl acetate.

The organic product stream will then be concentrated by thin film evaporation. The unit procedure has been specified to 99 % (molar) feed vaporization in 6 h. The solvent in the evaporated stream is assumed to be recycled to 90 % of a cost of 1.3 times the cost of energy for evaporation of the solvent. This is represented in the SuperPro Designer flowsheet as a component split (Figure 5.1).

5.2.3.5 Sensitivity analysis of the resting cell process

The parameters presented in Table 5.2 have been varied in the process model in SPD. As a base case, a yearly production of 5 tonnes has been assumed, an enzyme expression of 0.05 g/g cdw and an enzyme total turnover of 1 g substrate/g enzyme.

Table 5.2 Variable parameters and practical limitations. Bold numbers indicate the chosen base case values.

Part of process	Variable	Numbers	Motivation
Fermentation	Enzyme expression	0.01, **0.05**, 0.125 g/g cdw	Base case reached for the model system (1100 nmol/L). Maximum value chosen due to limitations of overexpression (Tufvesson et al. 2011).
Biocatalysis	Enzyme total turnover	0.1, **1**, 5 g substrate/g enzyme	1 g s/g corresponds to a substrate concentration of 10 mM in the base case.

5.2.4 Annual production

The target annual production has been set to 5 tonnes/annum, a reasonable number for pharmaceutical chemicals (Lima-Ramos et al. 2014). The process has been scaled in such a way that the number of batches has been decreased instead of the size of the equipment or any other alternative. However, for the lower numbers in the sensitivity analysis, the target production cannot be met within one year and therefore the production cost during one year has been used (corresponding to a production of 1 tonnes/annum in the case of low enzyme expression and 0.7 tonnes/annum in the case of low enzyme total turnover).

5.2.5 Influence of DSP

As mentioned above, the DSP after a concentrated product stream will vary between products and is therefore not considered in more detail. The desired purity of the final product will also determine required purification and further contribute to the cost. To see the overall picture a remaining DSP cost of 2x and 10x the initial extraction and evaporation has been considered.

5.2.6 Alternative operating mode: Growing cells

For the growing cell process, the biocatalytic reaction is assumed to proceed in the fermentation vessel for 8 h directly following the fermentation process with carbon source fed similarly to the resting cell process.

5.3 Results and discussion

5.3.1 Sensitivity analysis

5.3.1.1 Enzyme expression

Already when looking at the axis of the figures below, it can be concluded that it is high value molecules that are relevant for this type of processes. Approximate market value for a high value chemical is in the range of 500 $/kg, medium value products 100 $/kg and low value products in the range of 1 $/kg. From Figure 5.2 it can be seen that expression achieved with the CYP106A2 mutant of 0.05 g/g cdw corresponding to 1100 nmol/L is definitely in the low end of what is required to keep the production cost reasonable. Further increase in enzyme expression to what has been considered the biological limitation of an overexpressed protein (12.5 %) would decrease the production costs to half of the base case (480 $/kg main product (MP) instead of 1089 $/kg MP). A P450 expression of 11 % of the cdw has also been reached and is a desirable target (Pflug et al. 2007).

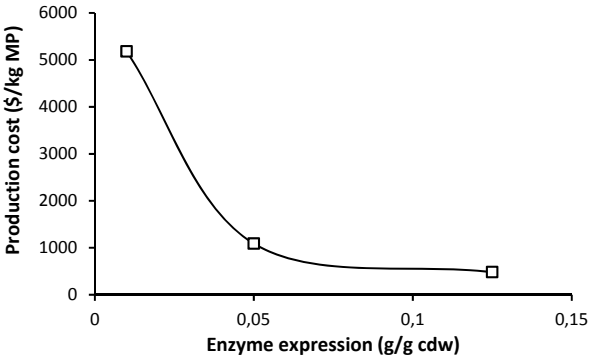


Figure 5.2 Influence of enzyme expression on the production cost.

5.3.1.2 Enzyme total turnover

After the biological limitation of the enzyme expression has been reached, the enzyme total turnover can be improved to lower the production costs. The conversion to the desired product is set to 90 % and the base case is assuming a substrate conversion of 1 g substrate/g enzyme within 8 h. In this case 1 g substrate/g enzyme corresponds to 0.05 g substrate/g cdw or 0.85 U/g cdw over 8 h. An improvement of 5 times in this variable would lower the production cost from 1089 $/kg MP to 275 $/kg MP (Figure 5.3). This increase to 4.25 U/g cdw is highly realistic also with respect to the cofactor regeneration capabilities of the whole cell, and could be increased even further based on findings in Chapter 2. However, this is calculated over 8 h and stresses the importance of the stability of the catalyst, which in the case of P450s might be a challenge. Further possible adjustments to the process that would lower the production cost that has not been considered here, is an increase in biomass concentration.

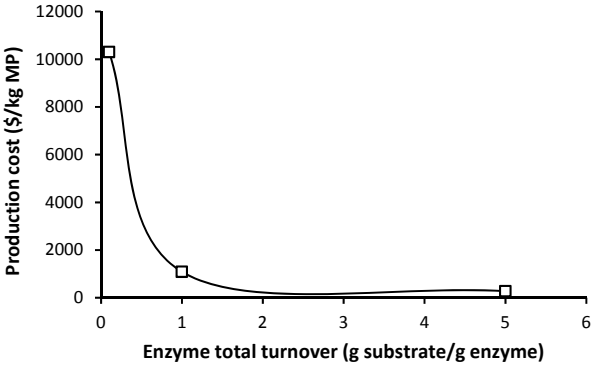


Figure 5.3 Influence of enzyme total turnover on the production cost.

5.3.2 Influence of DSP

In this chapter, data for the fermentation and biocatalytic process have been collected experimentally on a 2.5 L scale. However, the DSP is more uncertain and a liquid/liquid extraction has been assumed followed by evaporation to concentrate the product stream. No significant cost difference was found between recovery by extraction or adsorption using Amberlite XAD-7 resin in the recovery of 3-fluorocatechol from a biocatalytic process using fluorobenzene as substrate (Johnston et al. 1987), implying that this alternative also could be an option valid in this case. The variables applied for the sensitivity analysis have resulted in various product concentrations summarized in Table 5.3. Values for the stream after the transformation and the final product containing stream is presented. The resulting various product concentrations will also influence the cost of the required remaining DSP operations (Riet 1986; Datar 1986).

Table 5.3 Product concentration in different cases with an assumed annual production of 5 tonnes/year. Base case numbers are represented in bold. First row represents base case numbers, the following two rows represent variable enzyme expression and the lowest two represent variable enzyme total turnover.

Enzyme expression (g/g cdw)	Enzyme total turnover (g s/g cdw)	[P] after transformation (g/L)	[P] after evaporation (g/L)
0.05	**1**	1.3	184
0.01	**1**	0.3	41
0.125	**1**	3.4	387
0.05	0.1	0.1	21
0.05	5	6.6	468

The remaining DSP cost will highly influence the total production cost, therefore assumptions on the remaining cost has been made. Analyzing an itemized cost report, in the base case it can be seen that the DSP procedures accounted for here represents 23 % of the total production cost (Figure 5.4). The final purification is highly dependent on the type of product and in previous published fermentation processes (although not biocatalytic processes) the recovery costs was 50 % of the total costs for a penicillin product and 8 % for ethanol (Datar 1986), indicating that this range is also realistic for the products from P450 catalyzed processes. If the cost for DSP is doubled or even increased 10-fold the total production cost is considerably increased.

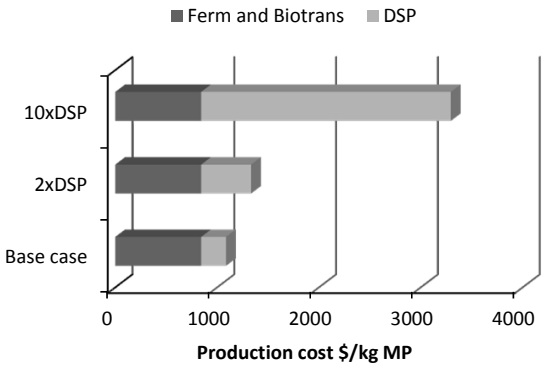


Figure 5.4 Influence of remaining DSP cost of the total production cost. The distribution between fermentation combined with biocatalysis and DSP is also illustrated.

In the process above, the product is assumed to be localized attached to the cells, thereby requiring extraction by organic solvent prior to cell separation preventing reusability of the cell. However, the product can potentially be accumulated inside the cells, attached to the cell membrane or present in the aqueous phase. For a product in the aqueous phase a first centrifugation step (using e.g. a decanter) to remove the cell mass is expected to simplify following DSP and allow reuse of the catalyst. Furthermore, extended experimental data for possibility of cell recycle and the consequence of the presence of cells during DSP is needed.

5.3.3 Operating mode

The analyses above have been made based on a resting cell process. A resting cell process gives the opportunity for more process optimization compared to a growing cell process and also the possibility of cell recycle. However, since the assumption has been made that the product is attached to the cell membrane, recycle of the cells is not possible and the resting cell process has therefore been compared to a growing cell process. The growing cell process excludes the diafiltration unit as well as the transformation vessel and the stream from the first fermentation vessel is transferred directly to the extraction unit. The fermentation step of the growing cell process has been assumed to be identical to the fermentation process in the resting cell process. The biocatalytic reaction is assumed to take place immediately after the fermentation, in the same vessel, with carbon source fed similarly

to the resting cell process. The base case production cost can with a growing cell process be decreased from 1089 $/kg MP to 756 $/kg MP (Figure 5.5). In the growing cell process, only one of the two available fermentation vessels has been utilized. The process could thereby be optimized further, by clever scheduling or introduction of a storage tank prior to DSP to decrease costs further.

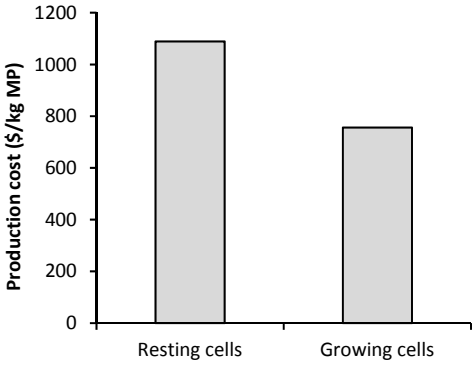


Figure 5.5 Comparison of resting and growing cells and the influence on the production cost.

5.3.4 Best case scenario

Combining a high expression and a high enzyme total turnover in a growing cell process would result in a unit production cost of 118 $/kg MP. In a similar cost assessment for the production of 3-fluoroveratrole by *Pseudomonasi* T-12, the continuous biocatalytic route resulted in a production cost of 100-200 $/kg MP, indicating that the value is reasonable (Johnston et al. 1987). A unit production cost of 118 $/kg MP would require an enzyme expression of 12.5 % of the cell dry weight and a whole cell activity of 4.25 U/g cdw over 8 h. Even with these improvements of the biocatalyst, it can be seen that the cost falls under production of pharmaceuticals or high value chemicals, above 100 €/kg corresponding to around 116 $/kg (Lima-Ramos et al. 2014). These combined improvements also results in a final product concentration after the combined fermentation and transformation step of 15 g/L, in the same order of magnitude of what has previously been presented as general guidelines for high value molecules in Chapter 2.

5.4 Conclusions

A process model has been set up in SPD using a fixed production plant of two fermenters with the size of 50 m^3 and DSP scaled accordingly including extraction to an organic solvent and evaporation to concentrate the product stream. The analyses in this study show that with realistic improvements to the P450 expressing whole cell catalyst, in terms of enzyme expression to 12.5 % of the cell dry weight and whole cell total turnover of 4.25 U/g cdw, production costs in the range of what is reasonable for pharmaceuticals and high value chemicals are within reach. It can also be seen that under the premises that the product is accumulated attached to the cell membrane, ruling out recycling of the catalyst, growing cells is the preferred operating mode.

6 Discussion

This chapter will connect the findings from the individual parts of the thesis, the initial literature based analysis with the two case studies presented in Chapters 3 and 4 together with the final cost assessment in Chapter 5. The findings will be discussed in relation to process development and implementation of P450 whole cell catalyzed processes. After commenting on the general methodology and suitable target products, the influence on catalyst, reaction and process related parameters will be discussed. Finally, suggestions for successful implementation of whole cell P450 catalyzed processes are presented.

6.1 General methodology

As emphasized repeatedly in scientific literature, P450s perform tempting chemistry to implement this group of enzymes for synthetic production at industrial scale. The novelty of this work has been to put the published literature in relation to demands on an economically feasible process, something that has been missing in the field. As stressed throughout this thesis, there are many challenges associated with the development of economically feasible P450 catalyzed whole cell processes. A general methodology has been applied to identify bottlenecks in whole cell catalyzed P450 processes. In the first part of the thesis (Chapter 2) economic metrics were applied to identify challenges within the field from literature data. The identified challenges have then been used as a basis for the case studies applied in the thesis. An important lesson is that development time and cost can be saved by *in silico* process design, meaning that it would be more efficient to spend time in front of the computer before entering the lab. For this analysis, the product of interest should be the starting point. After the product of interest has been identified, the overall synthesis route should be selected, and at this point P450s can be considered. A suitable P450 and substrate can be determined from literature and also the physical properties (e.g. solubility and logP guiding the toxicity) can be collected from literature. Already here potential reactor configurations and process options can be identified, although the growing or resting whole cell catalyst in combination with the oxygen demand speaks in favor of a stirred tank reactor. However, suitable modifications to this reactor could

be beneficial. Furthermore, a suitable host cell can be selected based on the knowledge gained from the *in silico* analysis. Challenges and potential limitations for reaching an economically feasible process identified from the first analysis can be divided into catalyst related, reaction related and process related parameters. Requirements on these parameters and potential improvements to the individual parameters were also identified in Chapter 2 and have then been the reference targets for the following case studies. All aspects are important for a successful development of these processes and as stressed in Chapter 2 (and Figure 2.5) the overall picture needs to be considered already at the beginning of the development.

6.2 Strategy for bottleneck identification and ranking

Throughout this work the order for theoretical and experimental identification of bottlenecks and order of importance have been attempted to be determined. However, it has not resulted in any conclusive answer. The result from this is instead that a whole cell P450 catalyst is a very complex machinery, also dependent on the outer environment such as parameters influenced by different reaction systems and process options. Taking biocatalyst yield for a resting cell process as an example, this is affected by inhibition, toxicity and specific activity, which in turn is dependent on enzyme expression, enzyme activity and stability of all components of the biocatalyst. Enzyme expression is the most independent parameter from the others that has a given target, guided by biological limitations for overexpression. All the other parameters are correlated and cannot be completely separated from each other. Toxicity and inhibition are also difficult to classify as catalyst dependent or reaction dependent since they are dependent on both mechanistic aspects of the catalyst but obviously also dependent of the molecule of interest.

6.3 Suitable target products

Suggested in Chapter 2, and confirmed in the cost assessment in Chapter 5, even for the best case scenario, the production cost of 118 $/kg of product indicates that it is high value product that is suitable for production by this type of catalyst and process. High value products include e.g. pharmaceuticals and their intermediates with a market value above 100 €/kg (Lima-Ramos, 2014). As discussed in Chapter 2, for products without competitive chemical processes, the market price of the

intended products can be a challenge to determine. The fact that there is not an established market does not imply that the price can be determined by the producer, an indication can still be received from the market or application of the product. Furthermore, for some types of products it might be a marketing advantage to have a biocatalytic production process.

6.4 Catalyst related parameters

Since P450s include more than 26 000 enzymes from all kingdoms of life there is a huge selection to choose from. However, far from all of them are suitable for overexpression and processes performed under economically feasible conditions. When the final research target is process development and industrial implementation, realistic improvements utilizing different tools need to be put in relation to economical feasible targets, as done during this thesis, to rule out non-suitable options before exploring the catalyst in the lab. Enzyme activities above 1 s^{-1} and expression levels around 12.5 % of the dry cell weight as well as a stable catalyst at relevant conditions are all parameters to strive for already in lab scale.

6.4.1 Cytochrome P450 monooxygenase

In the first case study, an artificial fusion construct was applied and expressed in the well-known laboratory strain *E. coli*. In the second case study, a multicomponent P450 was applied in a native P450 expressing host *B. megaterium* and finally a native fusion construct was applied in *E. coli*. In the two first cases, the stability of the correctly folded P450 was shown to be one of the main limitations for an economically feasible process. The poor stability of the enzyme could be due to many different reasons. The key for improvement of this trait is, therefore, to first identify the reason for inactivation. If the enzyme is worn out after a certain number of catalytic cycles, if certain amino acids are sensitive to oxidation or if poor coupling efficiency producing hydrogen peroxide being harmful to the active site is the cause, it has to be solved using different approaches.

6.4.2 Host cell

As presented in Chapter 2, impressive metabolic engineering work on entire pathways including P450s has previously been successfully implemented (Paddon et al. 2013, Szczebara et al. 2003). However, this requires enormous efforts and overexpression in well-established laboratory strains to

standardize and accelerate the development process of P450 catalyzed whole cell processes is tempting. It is, however, not that straight forward and the host cell selection in a P450 catalyzed whole cell process is of major importance for the process performance. The first selection criterion is related to the nature of the P450, whether a eukaryotic host cell is required or if a prokaryotic host is suitable. If the native P450 host is an option, this seems to be an advantage. The host is then capable of handling expression of heme-containing proteins (requiring δ-aminolevulinic acid) and is also adapted to cofactor requiring catalytic enzymes and can handle potential reactive oxygen species formed in the catalytic reaction.

The benefits of a native P450 expressing host were demonstrated in Chapter 4 where the cofactor regeneration by the resting *B. megaterium* was sufficient to support the catalytic reaction. In contrast, in Chapter 3, where *E. coli* was applied, the catalytic reaction was shown to be limited by cofactor regeneration and could be improved by addition of external NADPH. The overexpression of a cofactor regenerating dehydrogenase also confirms the indication of limitations by cofactor regeneration since lower P450 expression, when the dehydrogenase was expressed, did not negatively influence the reaction performance (Appendix 1, (Schewe et al. 2009)). The efficiency of the pentose phosphate pathway for cofactor regeneration of alternative heterologous hosts is also something that could be considered when selecting host. However, this was not considered in this thesis.

6.5 Reaction related parameters

None of the catalysts applied in this thesis (the gram positive *B. megaterium* or the gram negative *E. coli*) showed indications of transport limitations across the cell membrane for their respective substrates. It is hard to draw any general conclusions from this finding since only two substrates were applied (dodecanoic acid and CPA). However, it has been demonstrated previously for steroid substrates like CPA that the substrate transport across the cell membrane has been a limitation of a P450 catalyzed reaction performed in *E. coli* (Zehentgruber et al. 2010a).

Another reaction related challenge, highly relevant for mature P450 catalyzed reactions is the limited water solubility of the substrate. In the case of dodecanoic acid the low solubility could be used as an advantage for the process as a way to keep the dissolved concentrations low and thereby prevent

substrate inhibition. In the case of both dodecanoic acid and CPA, higher concentrations than applied in the studies would have required increased DMSO concentration (above 5 %). That would be detrimental for the enzyme, as already indicated at current conditions. This was not seen in the case of 4-ethylphenol but on the other hand potential loss of reactants by evaporation was introduced (Appendix 1). DMSO, or any other water miscible co-solvent, is commonly applied in research but should be avoided in full scale processes where it can make the DSP more difficult. For the assumed DSP in Chapter 5, where extraction followed by evaporation is applied, DMSO is remaining as the main impurity in the final product stream.

The other reaction related parameters mentioned in Chapter 2, inhibition and toxicity, are also dependent on the applied system. For all case studies inhibition by either substrate or product was shown to be limiting when aiming for high concentrations and economic feasibility. Inhibition of the P450 was, in the case of substrate inhibition by dodecanoic acid, partially circumvented by application of solid substrate and in the case of product inhibition by 15β-OH-CPA application of HP-β-CD resulted in a conversion of 98 %. Application of a second organic phase previously shown to be successful as substrate reservoirs or to enable ISPR (Schewe et al. 2009; Cornelissen et al. 2011), was in the case studies not successfully implemented. In the case of CPA conversion, the substrate was too hydrophobic to pass from the organic solvents tested to the aqueous phase and enable a reaction. Different reactor configurations e.g. with a circulating loop connected to the fermenter could enable several options for removal of product. By retaining the cells in the fermenter the biocompability of a solvent would be of minor importance as well as the explosion hazards caused by the aeration. Furthermore, in combination with a slow substrate supply to the fermenter, the general poor selectivity of the solvent between the substrate and product would also have limited importance.

6.6 Process related parameters

As discussed in this thesis, the requirement of cofactors, redox partners and relatively low stability of P450s makes whole cell catalyst the preferred catalyst form. To benefit from cofactor regeneration of the whole cell system, two process operating modes can be applied, growing and resting cells. As presented in Chapters 2 and 5, from an economical point of view, P450 catalyzed whole cell processes

are beneficial to run as growing cells. What could influence this statement is only if the catalyst could be recycled several times in a resting cell process or if the operating mode affects the product profile or any other crucial aspect of the catalyzed reaction. Recycling of the resting cell catalyst was shown not to be successful in Chapter 4 and does not oppose the statement. Furthermore, with the same model system, resting cells and growing cells were showing similar reaction performance in terms of product profile. The general differences between growing and resting cells would be interesting to explore further. The influence on the biocatalytic reaction caused by differences in the metabolic state would be very useful to establish since this could direct research efforts.

The best case scenario in Chapter 5, where growing cells are applied, assumes suggested improvements to the catalyst to be implemented as well as the assumption that these improvements would not lead to limitations by inhibition or toxicity. (Alternatively, if new limitations arise, that these can be addressed by process engineering.) The reasons for the advantage of growing cells in this assessment are that the catalyst concentration is kept identical to the fermentation in the resting cell transformation, the reaction performance is assumed to be identical between the growing and resting cell process and the cells are not assumed to be able to be recycled. This leads to fewer unit operations and shorter batch times for the growing cell process and thereby a lower production cost. The space-time yield of the improved growing cell process is, over the 48 h process and despite the assumed improvements, only 0.3 g/L/h, which according to the defined targets on this metric is not enough for economic feasibility. This is due to the relatively long time assumed for the fermentation process of 40 h followed by 8 h biocatalytic reaction. However, even if this was modified so that the biocatalytic reaction would be performed during the first 40 h the space-time yield would only be improved to 0.37 and would still not fulfill the requirement of 2 g/L/h, illustrating that this metric is a challenge to fulfill for growing P450 catalyzed reactions. To turn around the economic advantage of a growing cell process, one or more of the assumptions need to be proven wrong. The most challenging metric for resting cell processes is, on the other hand, biocatalyst yield, still assuming no cell recycle due to poor stability.

Another process related finding, presented in this thesis, is the differences between shake flasks and bioreactors, stressing the fact that sufficient mixing and proper oxygen supply is vital. Sufficient oxygen supply is of major importance for processes where the biocatalytic reaction as well as the catalyst requires oxygen. In practice during the three case studies, oxygen supply was never a problem when reactions were performed in bioreactors and oxygen supplied as air. However, increasing catalyst concentration to fulfill e.g. biocatalyst yields could require the air to be enriched with oxygen or adjustment of the carbon source feed rate in a growing cell process. Regardless, this is not considered to hinder the development of an economically feasible process.

6.7 Recommendations for implementation

The methodological approach implemented in this thesis has identified several bottlenecks for whole cell P450 catalyzed systems. Many of them are correlated, which is not only stressing the complexity of these systems, but also that several parameters needs to be addressed for successful implementation. The methodology was proven useful to identify targets for improvement and perhaps most importantly, to identify suitable products for this type of processes. The approach is something that is recommended to be applied in future development as well.

Except the scientific advices regarding targets and tools for implementation of whole cell P450 catalyzed processes, there are also “soft” skills required for successful implementation. In such a complex biocatalytic system, P450 experts cannot manage the development without support from other researchers such as protein engineers, metabolic engineers, fermentation specialists and process engineers. Communication between researchers with the involvement from interested companies is crucial for future development. Furthermore, understanding that P450s are challenging catalysts to implement is also essential while on the other hand the several “myths” created within the P450 community should be taken with skepticism.

7 Conclusions

Industrial biocatalysis is a promising alternative and complement to traditionally chemical catalyzed processes, emphasized by the increasing number of implemented processes (Nestl et al. 2011). P450s is one family of enzymes with a lot of potential for industrial biocatalysis. However, it has proven difficult to overcome the hurdles faced by P450s in order to reach an economically feasible process and thereby industrial implementation. Based on this thesis, general conclusions for successful implementation of P450 catalyzed whole cell processes can be drawn:

- Not all potential P450 products/processes are suitable for industrial application. Despite the large catalytic potential possessed by this family of enzymes, not all of them are suitable to implement for synthetic production at industrial scale. Realizing this is a first step to direct research efforts, which can be shown by an *in silico* analysis as done in Chapter 2.
- Given the challenges for the whole cell catalyst, focus should be on high value products to increase the success rate of implementation. This was confirmed by the economic assessment performed in Chapter 5, where an improved growing cell process resulted in a production cost of 118 $/kg product.
- The catalyst, the P450 and host cell, needs to be chosen wisely. Based on the desired catalytic reaction the P450 can be determined followed by selection of host, considering demands on biocatalyst-, reaction- and process- related parameters identified from the *in silico* analysis.
- Native hosts are beneficial for catalytic processes performed by P450s, since they can handle cofactor regeneration and expression of heme-proteins. This is exemplified in Chapter 3 where cofactor regeneration of the heterologous host *E. coli* was shown to limit ω-hydroxylation of dodecanoic acid. Meanwhile, in Chapter 4, the 15β-hydroxylation of CPA performed using the naturally P450 expressing host *B. megaterium* was not shown to be limited by the cofactor regeneration of the host cell.
- The stability of the P450 is crucial, as identified in the initial literature analysis (Chapter 2) and confirmed by the case studies (Chapters 3 and 4). In Chapter 3, 50 % of the correctly folded

P450 were lost after 2 h of reaction. Targets from the initial analysis were calculated on a 24 h process, and in the final cost assessment a 8 h process was assumed. If an increased stability cannot be reached, the specific activity, and all correlated parameters, needs to be improved beyond what was previously suggested to compensate for a shorter process time.

- P450 catalyzed reactions suffer from substrate and/or product inhibition. This was shown in the case studies at concentrations (0.43 g/L for ω-hydroxylation of dodecanoic acid and 0.23 g/L for CPA hydroxylation) far below industrial suitable targets of 20 g/L, and needs to be circumvented by engineering tools to enable higher concentrations.
- Solid supply of substrate can alleviate substrate inhibition, as demonstrated in the dodecanoic acid hydroxylation in Chapter 3. This approach improved the reaction performance from a final product concentration of 0.43 g/L to 1.2 g/L. Apart from the reduced substrate inhibition, it also avoids the negative effects on the biocatalyst caused by the co-solvent DMSO.
- *In situ* product removal is a promising method to tackle product inhibition. Chapter 4 shows that application of derivatized β-cyclodextrin to the 15β-hydroxylation of CPA by CYP106A2 expressed in *B. megaterium* circumvented the low substrate solubility and product inhibition, enabling an increase from 54 % to 98 % conversion of 1 mM substrate.
- Growing cell processes are the most economically suitable operating mode for whole cell P450 catalyzed processes. In the economic assessment in Chapter 5, the base case production cost was decreased from 1089 $/kg product to 756 $/kg product by changing operating mode from resting to growing cells. Further improvement in enzyme expression and enzyme total turnover and under the assumptions made in the analysis resulted in a production cost of 118 $/kg product for the improved growing cell process.

8 Future work

To continue exploring the field of whole-cell P450 catalyzed process development and enable industrial implementation, suggestions for further work that would improve and expand this thesis is presented in this chapter. Work aiming for development of the field in general is first presented followed by work recommended specific for each case studie.

8.1 General for whole-cell P450 catalyzed processes

8.1.1 Methodology development

The methodology used in the thesis with identification of bottlenecks divided in catalyst-, reaction- and process- related parameters could be expanded and improved. On the one hand, the methodology could be expanded to include any target molecule and a much greater selection of catalysts. On the other hand, a methodology more specific for P450s would make the results more accessible. To classify both reactants and P450s into groups, is one way to speed up the identification of bottlenecks for P450 catalyzed processes and thereby increase the rate of industrial implementation.

8.1.2 Platform host for redox catalysis

As stressed in Chapter 2 and shown throughout the thesis, the host cell should be selected carefully since it plays a vital role in the overall process. As already mentioned, a platform host for redox catalysis and more specifically P450 catalysis would be truly useful to speed up process development. This is not an easy task and also difficult to make general for P450s considering the various classes. However, even if a platform host is not reached, more widely spread common laboratory hosts adapted for cofactor requiring catalysis and synthesis of heme proteins would be a first step in this direction.

8.1.3 P450 stability

In the case studies in Chapters 3 and 4, the stability of the P450 was shown to limit the processes. This has a considerable impact on the potential of these types of processes. Therefore, it would be

interesting to study factors influencing the stability on a basic research level, to fully understand if the enzyme e.g. is worn out after a certain total turnover number or if it is directly correlated to the amount of hydrogen peroxide formed and thereby the coupling efficiency. The stability could potentially also be influenced by the operating mode, and a growing cell could potentially continue to synthesize enzymes and thereby keep a constant level of active enzymes over a longer period of time compared to resting cells.

8.1.4 Operating mode

From an economic point of view, growing cells have been shown to be the preferred operating mode. However, a more detailed comparison of how factors resulting from the different metabolic state of the cell influence the process in terms of selectivity and yields, and also enzyme stability and inhibition profile using different systems would be beneficial for further comparisons.

8.1.5 Approaches for substrate supply and product removal

Rising from the origin of enzymes and the feedback inhibition often involved in enzyme regulation, product inhibition is common in many biocatalytic reactions. Cost efficient and highly specific ISPR techniques would be beneficial also for P450 whole cell catalyzed processes. Nevertheless, since the substrate and product properties for typical P450 reactions are very similar, a successful ISPR is particularly challenging. As discussed in Chapter 6.5, modifications to a stirred tank reactor with e.g. an external loop would be one alternative approach to the commonly used one pot two-phase system and something recommended to be evaluated further.

8.2 Future work specific to case studies

8.2.1 Case study 1

Production of ω-hydroxylated medium and long chain fatty acids by a biocatalytic process is very interesting since the chemical route suffers from many drawbacks and the product has many areas of application including further functionalization. For demonstration of limitations of an overexpressed P450 in a non-natural host, the system worked well. However, for this type of product, the system applied in Chapter 3 is perhaps not the most suitable one. If the target product remains the same,

taking one step back and reconsider catalyst options and process alternatives is recommended. Further optimization of the whole cell process with CYP52 expressed in *Candida tropicalis* with already high product concentrations achieved is recommended instead (Lu et al. 2010).

8.2.2 Case study 2

15β-hydroxylation of CPA by CYP106A2 expressing *B. megaterium* is a promising process worth to investigate further. The application of HP-β-CD was shown to be successful, but there is room for improvement. Alternative cyclodextrins could also be considered, since non-derivatized options are significantly cheaper than modified ones. Furthermore, the process itself could be optimized in terms of ratio of HP-β-CD to CPA and actual CPA applied to the reaction as a complex.

8.2.3 Cost assessment

Depending on the purpose with a further cost assessment, different approaches can be taken. The field of biocatalytic process evaluation in general would benefit if a more generalized approach is chosen. From another perspective, in this specific case, the uncertainty is greatest in the DSP and this is something generally not considered in research laboratories despite having a major influence on the overall cost. Therefore, a systematic approach for potential DSP routes for different process options and product categories is recommended.

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Appendix 1 Supplementary information for the cost assessment in Chapter 5

A 1.1 Material and methods

A 1.1.1 Chemicals, Bacterial strains and plasmids

All used chemicals were from standard sources and of highest grade available. Solvents of analytical grade or HPLC grade were used for UPLC. *Escherichia coli* HMS174 (DE3) was purchased from MerckMillipore (Schaffhausen, Switzerland), pET22b used to insert CYP102A1 double mutant from Novagen (Merck,Darmstadt, Germany) and plasmid pCDF-1b from Novagen (Merck, Darmstadt, Germany) for insertion of cofactor regenerating glucose-6-phosphate dehydrogenase (G6PDH). Antibiotic resistance was used as selective marker, carbenicillin for the CYP102A1 double mutant and streptomycin for the G6PDH. The antibiotics were applied to the fermentation broth in final concentrations of 100 µg/mL (carbenicillin) and 50 µg/mL (streptomycin).

A 1.1.2 Fermentation

A seed culture with LB media supplemented with antibiotics was inoculated from a -80 °C glycerol stock and grown at 37 °C, 180 rpm until late exponential phase in shake flasks. The seed culture was used to inoculate a fermenter with 2.5 L working volume (new MBR, Zürich, Switzerland) and fermentation was performed by glucose limited fed-batch with a growth rate of 0.1 h^{-1} (Marisch et al. 2013). Set points controlled during the fermentation were pH set to 7.2, maintained by addition of 14 % NH_4OH and pO2 set to 30 %, controlled by agitation. Feed was initiated when the carbon source from the batch medium was depleted, indicated by a pO2 spike. Protein expression was induced by 0.5 µmol IPTG per g cdw one generation after feed start and the process continued for 3 additional generations. During the last generation, the feed profile was changed to a linear feed. Temperature was decreased at the time of induction from 37 °C to 30 °C. Foam was controlled by addition of 0.5 mL antifoam 204 (Sigma-Aldrich) per liter media. The final cell density reached was 33 g cdw/L.

A 1.1.3 Resting cell transformation

Cells were harvested by centrifugation (6000 rpm, 40 min) (HiCen XL, Herolab, Wiesloch, Germany) and washed with 1 L 100 mM potassium phosphate buffer (6000 rpm, 25 min) before the pellet was

resuspended in 2 L potassium phosphate buffer containing 1 % glycerol and 20 mM glucose. Resting cell biotransformations were performed in the same bioreactors as the fermentation with a working volume of 2 L. The transformations were initiated by substrate addition from a 1 M stock solution in DMSO after initial conditions had stabilized (30 °C, pO2 30 %, 1 vvm, pH 7.2 controlled by 20 % potassium hydroxide and 20 % phosphoric acid). During the transformation, carbon source was added from a stock mixture containing 27 % glycerol and 0.67 M glucose and a suitable linear feeding was determined from a resting cell biotransformation aiming at a pO2 of 30 % at 1 vvm resulting in a feed of 7 g/g cdw during 8 h. Cell density applied in resting cell transformation were 17 g cdw/L.

A 1.1.4 Growing cell transformation

When growing cells were applied as operating mode, the fermentation process was identical to the fermentation process for producing resting cells and the substrate (4-ethylphenol) was added when the linear feed was initiated, 3 generations after feed start.

A 1.1.5 Analytical methods

Cell dry weight

Samples for cell dry weight determination were analyzed using a halogen moisture balance (HG63-P, Mettler Toledo, Greifensee, Switzerland)

P450 determination

300 µL samples were collected from fermentations or transformations and pellets were frozen for P450 concentration determination by CO differential spectral assay (Omura and Sato 1964). Prior to analysis, samples were diluted with 100 mM potassium phosphate buffer to OD_{600} of 10. A spatula tip sodium hydrosulfite was added to the samples which were incubated on ice for 10 min before they were split into 3 wells of a 96-well plate, of which 1 was treated as blank and 2 were treated with CO during 1 min before the differential spectra was measured between 400 and 500 nm.

UPLC analysis

100 µL samples were taken for UPLC analysis. To stop the reaction, 10 µL HCl was added before diluting the samples with 500 µL solvent A (10 % acetonitrile in 0.05 % phosphoric acid) and 500 µL

acetonitrile. The supernatant was transferred to a fresh vial after centrifugation. Analysis was performed on an Acquity UPLC, with PDA detector, (Waters, Milford, MA, US) using a reverse-phase Acquity UPLC HSS T3 column (1.8 µM, 2.1x100 mm) (Waters, Milford, MA, US). An injection of 1 µL was followed by a gradient from 10 % to 90 % acetonitrile in 0.05 % phosphoric acid over 2.5 min. After 0.6 min at 90 % acetonitrile the gradient was returned to initial conditions for 1.7 minutes before the next injection. The flow rate was kept at 0.3 mL/min and the column oven was set to 40 °C.

A 1.2 Results and discussion

Resting cell biotransformations were performed with 10 mM substrate concentration, in batch and fed-batch mode (Figure A.1). The method of substrate supply did not influence the final result of the reaction and a reaction yield of 21-23 % was achieved. However, according to the analysis only 54 % and 69 % of the calculated added substrate can be found as substrate or product after 24 h of reaction, in the batch and fed-batch mode, respectively. For future work using this model system, the discrepancy should be investigate further and could be explained by analytical defects or by evaporation of the substrate and/or product. However, for the cost assessment a reaction yield of 90 % has been assumed.

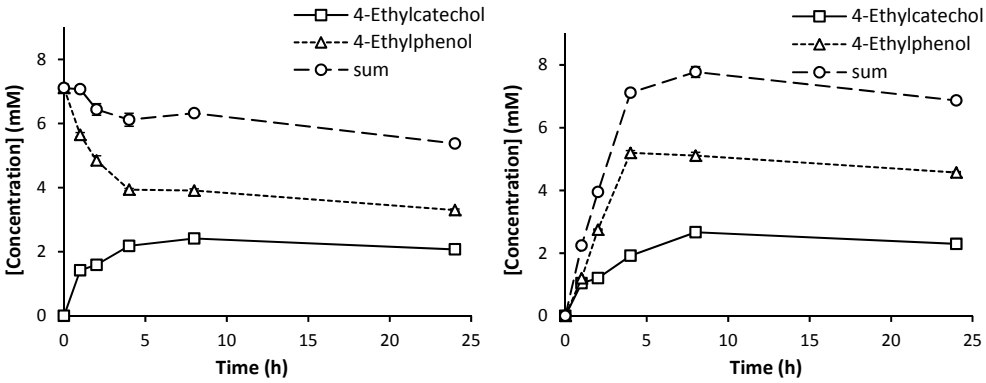

Figure A.1 Progress curves of biocatalytic reactions utilizing resting cells. Substrate supplied as 10 mM batch (left) and 10 mM fed-batch (right) with a linear feed profile over the initial 4 h.

An evaporation test was performed to determine whether this was the cause for the inconsistent mass balances seen in Figure A.1, using 5 mM 4-ethylphenol and 5 mM 4-ethylcatechol in 100 mM potassium phosphate buffer. Conditions during the evaporation test were identical to the resting cell transformations, except that no cells were added. Both the substrate and product was discovered to decrease over time (Figure A.2), with 70 % of the calculated value remaining 5 min after substrate and product addition and 58 % remaining after 24 h. The main loss was discovered to be allocated within 5 min after addition of the reactants to the transformation vessel, indicating that the analytical method needs to be improved or alternatively that there is a spontaneous fast reaction taking place. However, the decrease between 5 minutes and 24 h indicates that evaporation is also part of the explanation and mainly evaporation of the product 4-ethylcatechol.

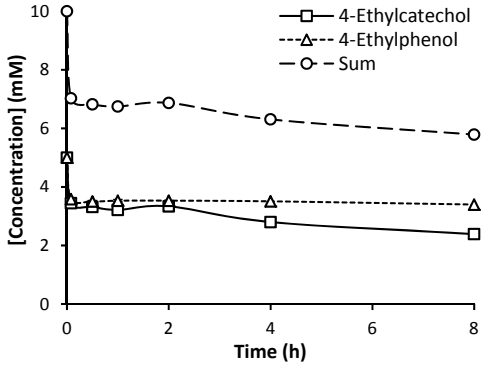

Figure A.2 Progress curve from evaporation test of 5 mM 4-Ethylphenol and 5 mM 4-Ethylcatechol. Points at time 0 illustrates calculated values.

In an attempt to increase the conversion in the resting cell biotransformation, repetitive batch supply of the substrate was applied. Furthermore, since the cofactor regeneration was shown to be a limiting factor in previous P450 catalyzed resting cell processes, glucose-6-phosphate dehydrogenase was overexpressed together with the CYP102A1 double mutant for improved cofactor regeneration. Neither the repetitive batch nor the coexpression of dehydrogenase improved the reaction performance compared to the initial biotransformation studies (Figure A.3). Limitation by the cofactor regeneration of the host cell in the initial resting cell biotransformation was confirmed in these

studies, since the overexpression of the dehydrogenase lead to a decrease in the P450 expression without changing the reaction performance (Figure A.3 and A.4).

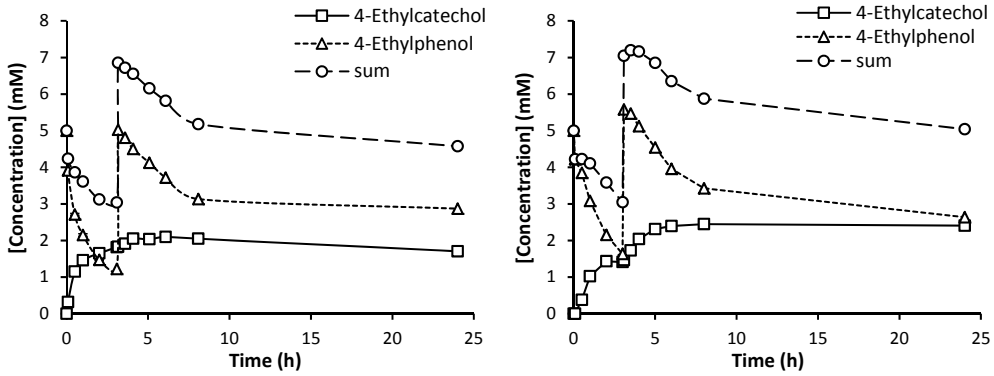

Figure A.3 Progress curves of biocatalytic reactions utilizing resting cells, with substrate supplied as repetitive batch (5+5 mM) from a 1 M stock solution in DMSO. Left graph illustrates *E. coli* expressing CYP102A1 double mutant and right graph illustrates *E. coli* expressing CYP102A1 double mutant and G6PDH.

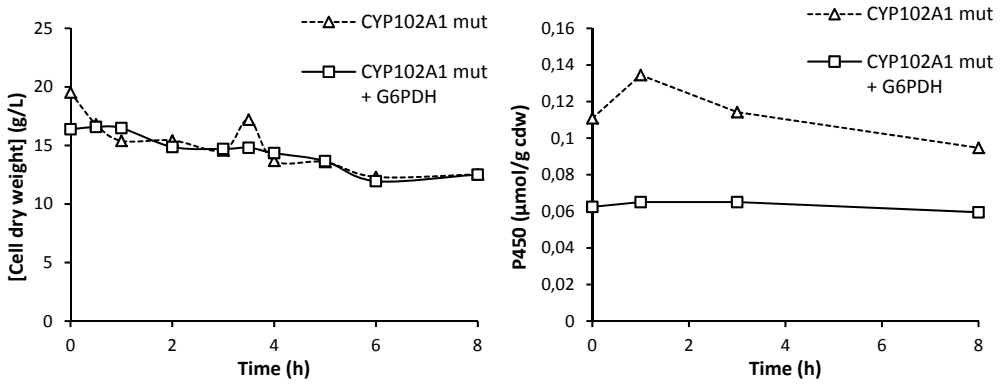

Figure A.4 Cell dry weight measurements and P450 concentration determination of biocatalytic resting cell reactions illustrated in Figure A.3.

To compare the two operating modes, resting and growing cells, a growing cell process was also performed. The repetitive batch supply of substrate was applied, 5 mM substrate was applied 3 times.

The conversion did not show any significant difference compared to resting cells (Figure A.5) and the same assumptions as for the resting cell process was made for the cost assessment with 90 % reaction yield over 8 h when applying 10 mM substrate.

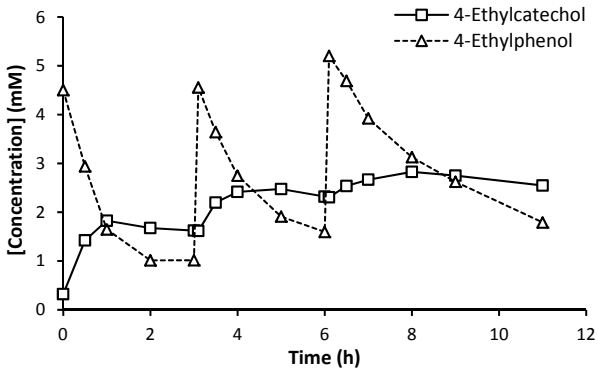

Figure A.5 Progress curve of biocatalytic growing cell reaction utilizing *E. coli* expressing CYP102A1 double mutant. Substrate supplied in a repetitive batch mode (3x5 mM).